Novel Mitotic Functions of Stem Loop-Binding Protein (SLBP)

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

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Abstract

Histone pre-mRNAs are the only animal mRNAs known to lack a polyA tail, instead ending in a conserved 16-nucleotide stem loop. Stem Loop-Binding Protein (SLBP) binds this structure, facilitating the processing into mature mRNA, and functioning in exporting this mRNA from the nucleus for translation. The loss of SLBP in humans results in Wolf-Hirschhorn Syndrome, highlighting the importance of SLBP function in cellular and developmental processes. Previous studies have shown that loss of SLBP in *Drosophila* leads to pleiotropic effects that have been attributed to DNA damage caused by a lack of histone protein. However, reducing histone levels themselves does not cause DNA damage, suggesting SLBP may have additional cellular functions. We have previously found that *Drosophila* SLBP localises to the spindle area during mitosis and that it biochemically associates with a large number of proteins involved both in translational control, and in cell cycle regulation. In this study I investigate the possibility that SLBP functions in mitosis in the early *Drosophila* embryo. Using RNAseq I demonstrate that SLBP does not differentially associate with the mRNAs of the interacting proteins. In contrast, I show that loss of SLBP results in mis-localisation of at least some of these interactors; suggesting that, rather than regulating their translation, SLBP is involved in directly regulating the function of these proteins. I also show that RNAi of SLBP in the embryo results in a wide variety of problems which can be classified as a DNA damage-like response; however, these defects can occur in the absence of DNA damage. I suggest a hypothesis for how SLBP functions to control so many proteins with such a wide variety of functions, through co-ordination with the DNA damage response checkpoint kinase 2 (CHK2).

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List of Abbreviations

APC	Anaphase-promoting complex
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia
ATRIP	ATR-interacting protein
CA-CHK2	Constitutively active CHK2
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CNN	Centrosomin
DSB	Double-stranded break
EB1	End binding protein 1
ESP1	Extra spindle pole bodies
GDP	Guanosine diphosphate
GRIP84	Gamma ring protein 84
GRIP91	Gamma ring protein 91
GTP	Guanosine triphosphate
H2AV	Histone 2A variant
IP	Immuno-precipitation
MAP	Microtubule-associated protein
MS	Mass spectrometry
MT	Microtubule
MUD	Mushroom body defect
PCM	Pericentriolar material
PDS1p	Precocious Dissociation of Sisters
PINS	Partner of Inscuteable
PLK1	Polo-like kinase 1

RPA	Replication protein A
SAC	Spindle-assembly checkpoint
SCC1	Verthandi
SCC3	Stromalin
SLBP	Stem loop-binding protein
SMC1	Structural maintenance of chromosomes 1
SMC3	Structural maintenance of chromosomes 3
WHS	Wolf-Hirschhorn Syndrome
γ-tub	Gamma tubulin
γ-TuRC	Gamma tubulin ring complex
y-TuSC	Gamma tubulin small complex
+TIPs	Plus end binding protein

1. Introduction

It is of vital importance to living organisms that the cell cycle and mitosis function correctly. Most cells possess a number of checkpoints, monitoring correct progression through different stages of the cell cycle and responding to defects accordingly, either through arresting the cell cycle or through activating apoptotic or related programmes. Disruptions to mitosis in cellular systems with weak checkpoints, or those in which checkpoint activity, or the resultant response, is lost, can lead to disease, including cancer. Therefore, it is essential to identify the proteins that function during mitosis and how they are regulated, both in normal cells and in disease models.

1.1 The cell cycle

1.1.1. Stages of and progression through the cell cycle

The cell cycle consists of 2 stages: interphase and mitosis. Interphase consists of S-phase, in which the genome is accurately replicated, separated by 2 'gap' phases (G_1 and G_2), in which proteins necessary for cell function and cell cycle transitions are produced (Wu, Liu and Kong, 2014; Wu *et. al.,* 1993). Mitosis is the process of dividing replicated DNA into 2 equal cells. In Eukaryotic organisms, progression through the cell cycle is regulated through the activation and inhibition of cyclin-dependent kinases (CDKs) (reviewed in Suryadinata, Sadowski and Sarcevic, 2010) (Fig.1-1). This is essential to ensure that cells co-ordinate DNA replication with chromosome segregation, preventing uncontrolled division; a known cause of cancer.

CDKs were first described by Paul Nurse (1975) after experiments in the fission yeast *Schizosaccharomyces pombe* identified temperature-sensitive mutants in which cells divided abnormally. CDKs work in conjunction with a class of

proteins called cyclins. Cyclins were first discovered by Evans *et. al.* (1983), through observing sea urchins they identified proteins whose abundance consistently changed during the cell cycle. Further research on *Xenopus laevis* oocytes determined that addition of *Spisula* cyclin A mRNA was sufficient to induce meiosis and that both cyclins A and B were necessary for entry to mitosis (Westendorf, Swenson and Ruderman, 1989; Swenson, Farrell and Ruderman, 1986). The nomenclature for cyclin and CDK proteins is variable between organisms. For the purposes of this introduction I will refer to the names given to the human proteins.

In humans, concentrations of CDKs remain constant throughout the cell cycle and their activity is regulated through binding by cyclins. Cyclins activate CDKs to phosphorylate target substrates required for cell cycle progression. Different CDK-cyclin combinations form at different stages, with each one promoting the activation of the next in the sequence (Fig.1-1). CyclinD/CDK4-6, CyclinE/CDK2, CyclinA/CDK2, CyclinB/CDK1 combinations mediate, respectively, entry into G_1, G_1 to S-phase transition, progression through G_2 and entry into mitosis (reviewed in Gérard and Goldbeter, 2009; Nurse, 2002). Once cells have passed the G_2/M transition into mitosis, this cannot be reversed (Stark and Taylor, 2004). There are 3 main ways in which this process is controlled: targeted protein destruction, phosphorylation and CDK inhibition (De Luca *et. al.,* 1997; Dowdy *et. al.,* 1993; Harper *et. al.,* 1993).

Mitosis consists of 4 stages: prophase, metaphase, anaphase and telophase. During prophase, duplicated chromosomes begin to condense and the nuclear envelope generally breaks down (Smoyer and Jaspersen, 2014; Vagnarelli,

2012). In metaphase, condensed chromosomes align at the metaphase plate through the co-ordinated force elicited by the microtubule (MT)-based mitotic spindle, which attaches to specialised proteinaceous sites on the chromosomes, kinetochores (Guo, Kim and Mao, 2013). Once all chromosomes are correctly aligned, and the so-called spindle assembly checkpoint (SAC) has been satisfied, the cell progresses into anaphase. Here the kinetochore-MTs depolymerise, while maintaining their attachment to the kinetochores, resulting in chromatid movement towards the spindle poles (Nasmyth, 1999). Finally, in telophase, once chromatids have reached opposite poles, the nuclear envelope re-forms and chromosomes de-condense. Upon cytokinesis, this results in the formation of two genetically identical daughter cells (Collas, 1998).

The cellular machinery that carries out mitosis, and the processes governing it, will underpin my research in this project.

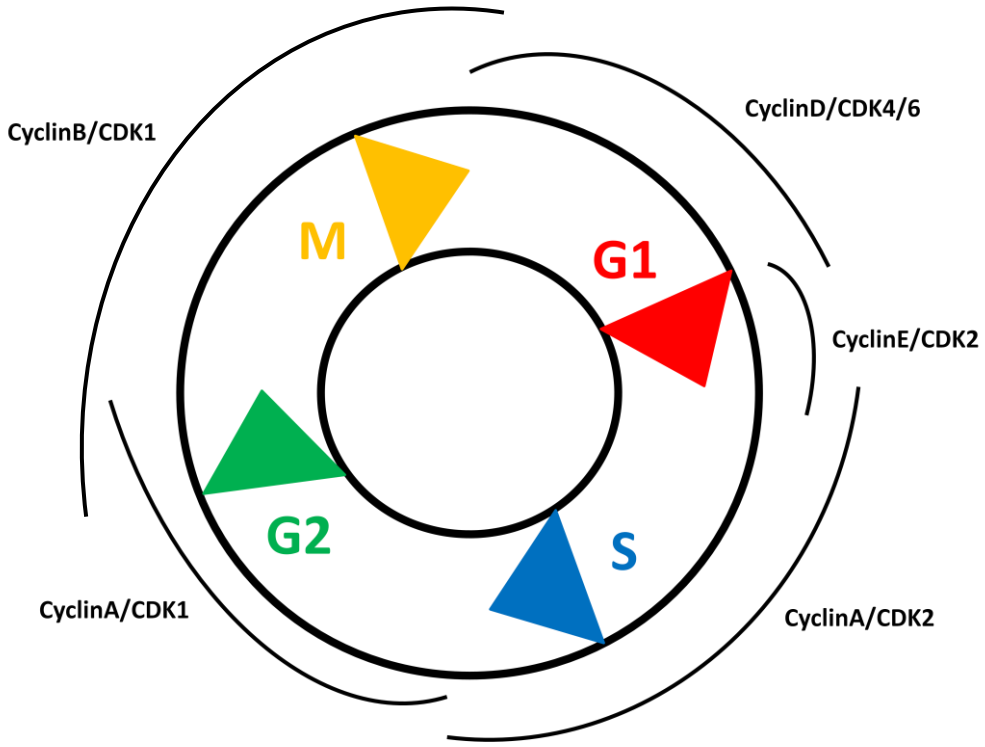


Figure 1-1. Cyclins and CDKs through the cell cycle. At the beginning of interphase, cyclin D is expressed and binds CDK4/6. This leads to cyclinE/CDK2 formation, mediating progression through the G_1/S transition. Through S-phase and into G_2 cyclinA/CDK2 complexes form, before expression of cyclinB and activation of CDK1, which mediates entry into mitosis. (Adapted from Suryadinata, Sadowski and Sarcevic, 2010)

1.1.2. Chromosome cohesion

Before duplicated chromatids are physically separated at anaphase, it is important that they remain joined. This is achieved through chromosome cohesion via the cohesin complex (reviewed in Haering and Jessberger, 2012). The cohesin complex consists of 4 proteins, Structural maintenance of chromosomes 1 (SMC1), SMC3, Verthandi (SCC1) and Stromalin (SCC3). SCC1 binds the head domains of SMC1 and SMC3, which then incorporates SCC3 (Anderson *et. al.,* 2002; Haering *et. al.,* 2002).

The cohesin complex was originally identified by Guacci, Koshland and Strunnikov (1997) and Michaelis, Ciosk and Nasmyth (1997) through genetic screens designed to discover proteins required for sister chromatid cohesion. It is believed to bind duplicated chromosomes by trapping them within the ring-like structure it forms, since research has shown that site-specific cleavage of SCC1 or SMC3, opening the ring-like structure, is sufficient to release sister chromatids (Ivanov and Nasmyth, 2007; Gruber, Haering and Nasmyth, 2003; Uhlmann *et. al.,* 2000; Waizenegger *et. al.,* 2000). Conversely, it has been shown that bonding cohesin to form the ring-like structure, traps circular sister DNAs (Haering *et. al.,* 2008).

However, cohesion needs to be released to allow sister chromatids to move to opposite spindle poles. This is achieved in 2 steps: first, removal of cohesin from chromosome arms in prophase, then, removal from centromeres in anaphase (Waizenegger *et. al.,* 2000). Removal of cohesin is mediated by the anaphase-promoting complex (APC) via ubiquitination of a target protein (Cohen-Fix *et. el.,* 1996); a process best understood in budding yeast. In yeast, the APC targets Precocious Dissociation of Sisters (PDS1p), an anaphase-inhibitor, releasing Extra Spindle Pole bodies (ESP1) which can go on to allow removal of cohesin from chromosomes (Ciosk *et. al.,* 1998; Cohen-Fix *et. el.,* 1996). Removal of cohesin requires cleavage of SCC1 (Uhlmann, Lottspeich and Nasmyth, 1999). More recently, Esp1 and its orthologs have been classified as separins and Psp1p and its orthologs have been classified as securins, to reflect their roles (Yanagida, 2000). Although SCC1 is the target for ESP1p, all cohesin subunits are essential for cohesion. Studies have shown that mutants for any component cause separation of sister chromatids in the absence of APC

activity (Tóth *et. al.,* 1999; Guacci, Koshland and Strunnikov, 1997; Michaelis, Ciosk and Nasmyth, 1997).

This process is highly conserved. Orthologs for Smc1, Smc3, Scc1 and Scc3 have been found in the cohesin complex in Humans (Sumara *et. al.,* 2000).

1.1.3. Microtubules and microtubule-associated proteins

Microtubules (MTs), an essential component of a cell's cytoskeleton, are vital during mitosis as they form the spindle apparatus that exerts force upon the duplicated chromatids, co-ordinating both their alignment at metaphase and their segregation at anaphase (Fuge, Bastmeyer and Steffin, 1985). They are formed from dimers of two related and highly conserved proteins, α- and β-tubulin, which self-organise end-on-end into protofilaments; 13 of these protofilaments associate laterally to generate a hollow, cylindrical structure - an individual microtubule (Fig.1-2) (Jacobs and Cavalier-Smith, 1977; Amos and Klug, 1974).

Microtubules must be dynamic to be able to generate a spindle capable of chromosome segregation. Their dynamic nature is conceptualised by the term 'dynamic instability' - first described by Mitchison and Kirschner (1984). α/β-tubulin heterodimers incorporate 2 GTP molecules. The GTP molecule is less tightly bound on the β-tubulin, and so is accessible to de-phosphorylation through the intrinsic GTPase activity of the tubulin molecule, forming GDP. Hydrolysis to GDP within the β-tubulin causes a 3D conformational change to the heterodimer, making the lateral interactions between neighbouring molecules within a MT less stable. Incorporation of GTP-tubulin dimers into pre-

existing MTs results in MT growth. Thus, the rate of addition of new GTP-tubulin dimers, in relation to the rate of tubulin-GTP hydrolysis to GDP-tubulin, is crucial. If the rate of addition exceeds the rate of hydrolysis, a GTP-cap will be maintained at the end of the MT, resulting in growth. If, however, the rate of hydrolysis is greater than the rate of addition of GTP-tubulin, the GTP-cap will be lost and the presence of GDP-tubulin at the end of the MT will trigger "catastrophe", and result in MT shrinkage. Once the critical local concentration of GTP-tubulin increases, the MT will be "rescued" and growth resume. (Fig.1-3) (Tran, Joshi and Salmon, 1997). This trademark of dynamic instability in MTs is highly conserved, indicating the importance of this function (Desai and Mitchison, 1997).

MTs are polar structures, which is essential to their function. α-tubulin is exposed at the minus ends of MTs, whereas β-tubulin is exposed at plus ends. Since the GTP molecule is more tightly bound by α-tubulin, the minus end of MTs remains fairly stable, while the plus end, which contains GTP that is more accessible for de-phosphorylation, is much more dynamic (Caplow and Shanks, 1995; Mitchison, 1993).

The sites of MT nucleation within the cell, the precise dynamics they exhibit, the cellular structures they interact with, and their co-ordinated organisation into specific forms are all determined by microtubule-associated proteins (MAPs). MAPs were first discovered in mammalian brain tissue by Borisy *et. al.* (1975) where a subset of proteins stabilise neuronal MTs, in order to maintain cell shape, and therefore function. However, MAPs are now generally regarded as any protein that associates with MTs, including those that function directly to

affect MT dynamics, that carry cargoes along MTs to specific sub-cellular sites, and those that associate with MTs in order to concentrate their function (Hughes *et. al.,* 2008; Maiato, Sampaio and Sunkel, 2004).

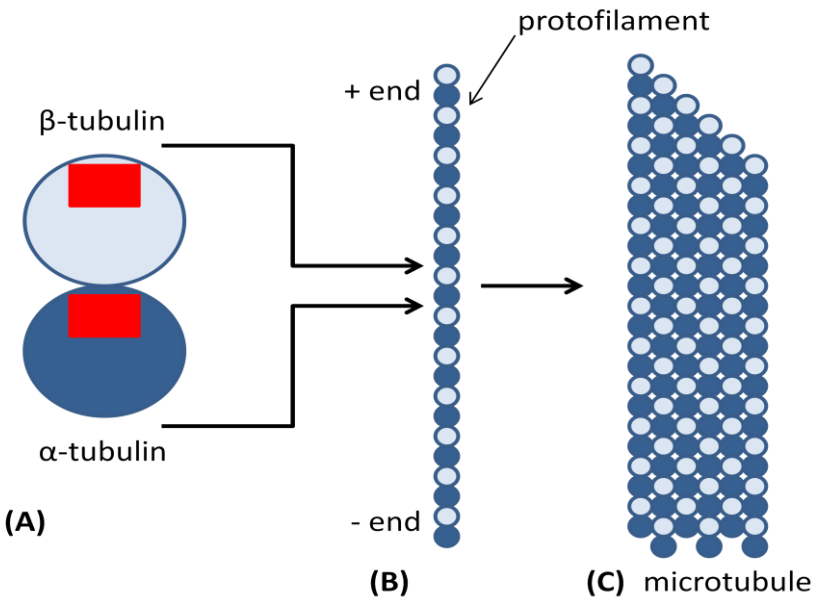


Figure 1-2. Composition of microtubules. (A) Individual heterodimers consist of an α- and a β-tubulin subunit. GTP nucleotides shown in red. (B) These heterodimers stack on top of each other to form alternating α/β-tubulin protofilaments with polarity. (C) A microtubule consists of 13 of these protofilaments arranged in a cylindrical structure. (Adapted from Alberts *et. al.,* 2008)

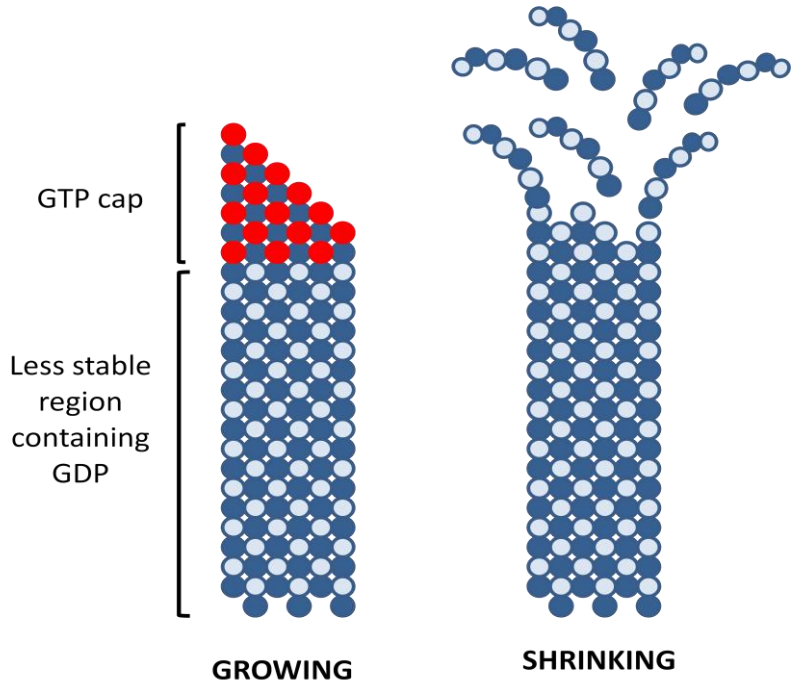


Figure 1-3. Dynamic instability of microtubules. Presence of a GTP-cap allows growth of microtubules by stabilising the linear conformation of protofilaments (red molecules represent GTP-β-tubulin). If the GTP-cap becomes de-phosphorylated to GDP, protofilaments lose their linear conformation, become curved and break away from the microtubule, causing catastrophe. (Adapted from Alberts *et. al.,* 2008).

1.1.4. Microtubule nucleation

During mitosis in animal cells, MT nucleation primarily occurs at the centrosomes (Varmark, 2004). Centrosomes consist of two perpendicular centrioles which organise the surrounding mass, called the pericentriolar material (PCM) (Bobinnec *et. al,* 1998). The PCM is able to nucleate MTs through concentrating a third member of the tubulin family, γ-tubulin (γ-tub), to the PCM, in the form of a structure called the γ-tubulin ring complex (γ-TuRC), which provides a template from which microtubules can grow (reviewed in Kollman *et. al.,* 2011).

The γ-TuRC is composed of γ-tubulin and at least 6 other proteins (reviewed in Gunawardane et al, 2000), referred to as gamma ring proteins (grips) (Zheng *et. al.,* 1995). Although this seems to be the preferred MT nucleator, a sub-complex, the γ-tubulin small complex (γ-TuSC) - consisting of γ-tubulin and two of the Grips - is able to nucleate MTs *in vitro.* In *Drosophila* the γ-TuSC is composed of 2 γ-tubulin molecules, together with DGRP84 and DGRIP91 (Oegema *et. al.,* 1999) (Fig.1-4).

At the onset of mitosis in humans, γ-TuRCs are recruited to centrosomes with the help of the centrosomal protein Pericentrin, resulting in vastly increased MT nucleation (Zimmerman *et. al.,* 2004). In *Drosophila,* the Pericentrin ortholog, D-PLP, works in conjunction with another protein, Centrosomin (CNN), to recruit the *Drosophila* γ-TuRC (Martinez-Campos *et. al.,* 2004). However, in fly embryos, this recruitment is not cell-cycle dependent; instead high levels of γ-TuRC are present at centrosomes prior to mitosis (see section 1.2.).

Although the centrosomal accumulation of γ-TuRC biases mitotic MT nucleation from centrosomes, therefore ensuring that, in cells that possess these organelles, they are the primary site of mitotic spindle assembly, the spindle can be generated by other MT sources; including: those initiated at chromosomes during mitosis, at kinetochores, and from within the spindle itself, through augmin-mediated MT generation (Duncan and Wakefield, 2011). Indeed, if functional centrosomes are removed from cells that normally contain them, these other pathways of MT generation compensate. This has been displayed in *Drosophila* cnn null mutants, where active centrosomes fail to form but flies survive until adulthood (Megraw, Kao and Kaufman, 2001). Pathways are

interlinked and this generates the flexibility of spindle formation (Hayward *et. al,* 2014).

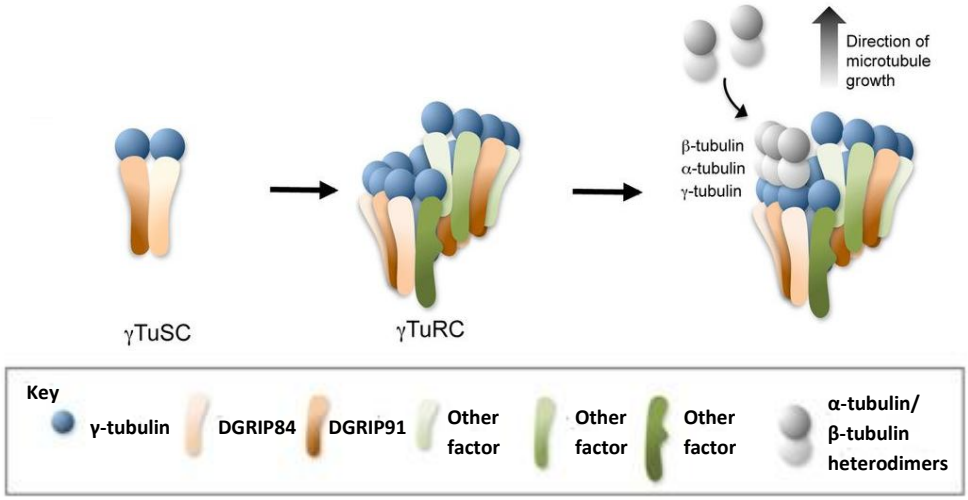


Figure 1-4. Microtubule nucleation through the γ-TuRC. A tetramer of DGRIP84, DGRIP91 and two γ-tubulin molecules combine to form the γ-TuSC. This tetramer combines with other factors to form the characteristic ring-like structure of the γ-TuRC which forms a template from which α/β-tubulin heterodimers can attach and initiate microtubule growth. (Adapted from Teixidó-Travesa, Roig and Lüders, 2012).

1.1.5. Centrosome duplication

In cells containing centrosomes, it is essential that they duplicate exactly once per cell cycle, so that a bipolar spindle can form. Failure to control centrosome duplication can result in multipolar spindles causing mis-segregation of chromosomes that can lead to an imbalance of proper control mechanisms and potential loss of tumour suppressor genes, which can contribute to uncontrolled growth and cancer (reviewed by Brinkley, 2001). Multiple duplications can result in a multipolar spindle. Although *de-novo* centrosome generation is possible, duplication of centrosomes generally relies on templated-duplication of centrioles (reviewed in Firat-Karalar and Stearns, 2014).

Centrioles are cylindrical structures usually composed of 9 triplet MTs (though centrioles in *Drosophila* embryos are composed of 9 doublet MTs, while centrioles in *Caenorhabditis elegans* sperm cells are composed of 9 singlet MTs (Dong, 2015)). Centrioles exist in pairs, as a mother and daughter centriole, that align perpendicular to one another; the mother centriole being one generation older than the daughter. As well as forming centrosomes, centrioles are also essential in the formation of cilia and flagella (Garcia-Gonzalo and Reiter, 2012; Dutcher, 1995). Cilia and flagella have essential functions in locomotion and sensory roles. Defects in cilia formation or function result in a class of diseases called 'Ciliopathies' (reviewed in Bachmann-Gagescu, 2014).

The molecular composition of centrioles has recently become much better understood, when studies on *C.elegans* discovered 5 core components: ZYG-1 (O'Connell *et. al.,* 2001), SPD-2 (Kemp *et. al.,* 2004; Pelletier *et. al.,* 2004), SAS-4 (Kirkham *et. al.,* 2003; Leidel and Gonczy, 2003), SAS-5 (Delattre *et. al.*, 2004) and SAS-6 (Leidel *et. al.,* 2005; Dammermann *et. al.,* 2004). These components are recruited in a hierarchical manner (Fig.1-5) (Delattre, Canard and Gönczy, 2006). This process has been found to be more complicated in other organisms, with recruitment of further proteins (Fig.1-5).

In most cells the duplication of centrioles begins in S-phase when mother and daughter centrioles separate slightly, stimulating formation of new daughters. In G_2 centriole pairs split and accumulate PCM. Each centrosome migrates to opposite poles of the cell at the onset of prophase, and after nuclear envelope

breakdown (NEB) begin to nucleate astral microtubules, using γ-TuRCs (Sluder and Khodjakov, 2010).

Multiple factors are involved in the regulation of this process (reviewed in Sluder and Khodjakov, 2010). For example, the disjoining of mother and daughter centrioles is believed to occur through the action of Polo-like kinase 1 (Plk1) in early mitosis and separase during the metaphase/anaphase transition (Tsou *et. al.,* 2009). This separation is believed to license the centrioles to replicate during the following interphase.

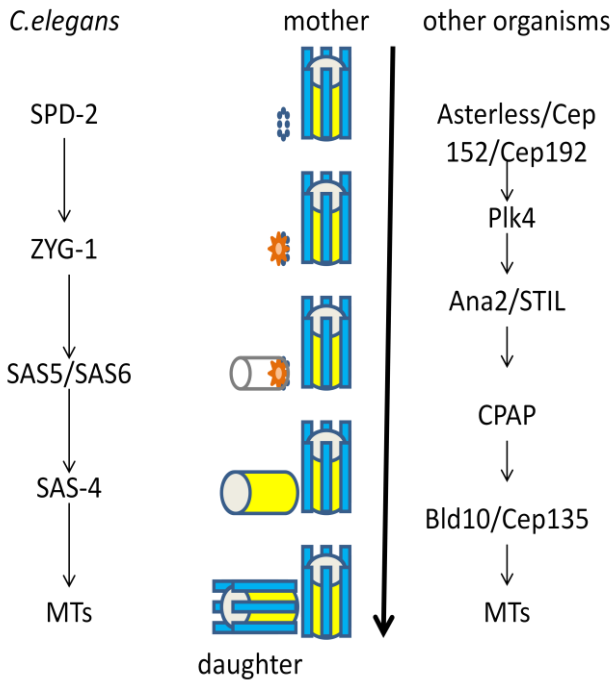


Figure 1-5. Centriole duplication. Formation of a new daughter centriole in *C.elegans* begins with recruitment of SPD-2, which leads to incorporation of ZYG-1. The SAS-5/SAS-6 complex is then recruited, forming the inner tube. This initiates incorporation of SAS-4, forming the outer tube, and finally 9 singlet microtubules form around the structure. On the right hand side are some of the other major proteins involved in this process in other organisms. (Adapted from Dong, 2015).

1.1.6. Cortical attachment and spindle orientation

Spindle orientation occurs through both mechanical forces and molecular level regulation (reviewed in Nestor-Bergmann, Goddard and Woolner, 2014; Lu and Johnston, 2013). Correct orientation of the mitotic spindle in relation to the cell cortex is essential for polarised divisions, important in embryos for tissue development (Baena-López, Baonza and García-Bellido, 2005) and in adults to prevent cancer (Pease and Tirnauer, 2011). This orientation requires connections from the plus-ends of astral microtubules to the cortex of a cell. The connection of the cortex and astral microtubules allows pulling forces to be exerted on spindle poles, bringing them into line (Kotak, Busso and Gönczy, 2012).

This occurs through plus-end binding proteins (+TIPs), such as End binding protein 1 (EB1) in *Drosophila* which ensures correct spindle orientation via stabilisation of spindle structure. Loss of EB1 in mitotic *Drosophila* S2 cells causes shortened astral microtubules and detached spindle poles (Rogers *et. al.,* 2002). EB1 also recruits other +TIPs which contribute to spindle orientation (Kumar and Wittmann, 2012; Slep, 2010). These +TIPs act as ‘spindle capture prey’.

The cortex contains positioning cues that organise other proteins to act as ‘spindle capture bait’, such as Partner of inscuteable (PINS) in *Drosophila* which organises the MUD-dynein complex (Lu and Johnston, 2013). Gαi, a G-protein subunit, binds to the plasma membrane and serves and an anchor for PINS. PINS incorporates Mushroom body defect (MUD) (the ‘prey’), which is bound to the dynein/dynactin complex; providing a link to microtubules and providing the

pathway for motor activity necessary to generate a pulling force (Bergstralh and St Johnston, 2014).

Multiple pathways exist for spindle to cortex attachment, this is just one example (more can be found in Lu and Johnston, 2013).

1.2. The *Drosophila* embryo as a model organism

Drosophila melanogaster is an ideal model organism in which to study the cell cycle and mitosis. Indeed, *Drosophila* have been used as a model organism for mitosis research for decades (reviewed in Yanagida, 2014). The mitotic apparatus in *Drosophila* is very similar to that in human cells, and about 60% of genes in humans that can result in disease, have homologues in flies (Schneider, 2000). In general it makes a good model because it has a rapid life cycle, an easily manipulated genome and is easy to maintain. In addition, embryos are amenable to microscopy techniques and available in large quantities for biochemistry. In the context of mitosis, flies are a good choice due to the initial rounds of DNA replication and division occurring as a syncytium. This was first characterised by Foe and Alberts (1983), who observed the features of the final 5 cycles prior to cellularisation. Initial rounds occur within the core of the embryo, but around cycle 9/10 nuclei migrate to the cortex, where they can be easily visualised using confocal microscopy. Divisions occur in waves originating at the poles of the cell and travelling in towards the midregion. At around cycle 14 cellularisation occurs. The initial divisions happen rapidly, approximately every 10-20 minutes, consisting only of recurrent S phases and mitoses, without intervening G phases (Foe and Alberts, 1983). This makes imaging of mitotic rounds quick; however, can result in high levels

of DNA damage which the embryo must respond to. This is carried out via a process called nuclear fallout, described in more detail below.

1.3. The DNA damage response

Regulation of DNA replication and mitosis is important to prevent DNA damage occurring, however, it can still happen and cells have a response in place for this (reviewed in Ciccia and Elledge, 2010).

Both single stranded (ss-) and double stranded (ds-) DNA breaks can occur. ssDNA breaks initiate recruitment of replication protein A (RPA) which in turn recruits the sensor kinase ataxia telangiectasia and Rad3-related protein (ATR) and bound ATR-interacting protein (ATRIP) (Cimprich and Cortez, 2008). dsDNA breaks can be recovered from using ATR after binding by the MRN complex, which holds the two strands together until they can be fixed (van den Bosch, Bree and Lowndes, 2003). However, ds-breaks can also be repaired by recruitment of another sensor kinase, ataxia telangiectasia mutated (ATM) (Suzuki, Kodama and Watanabe, 1999).

In vertebrates, the primary response to DNA damage is mediated by checkpoint kinases CHK1 and CHK2. CHK1 works as and effector kinase to the sensor ATR, and CHK2 works as an effector kinase to the sensor ATM (Smith *et. al.,* 2010). CHK1 and CHK2 become activated by phosphorylation when they are recruited to sites of DNA damage and go on to phosphorylate targets that mediate the response, either to repair the damage or arrest and destroy the cell.

In the latter case, to prevent cells entering a new cell cycle, CHK1 phosphorylates CDC25A, targeting it for degradation (Jin *et. al.,* 2008). CDC25A is required to de-phosphorylate the CDK2/cyclinA/E complex; therefore it's destruction blocks progression through the G_1/S restriction point. In addition, CHK2 activates p53, which induces cell cycle arrest and apoptosis by transcriptionally regulating the CDK inhibitor p21 and pro-apoptotic BAX and PUMA proteins, among other routes (Riley *et. al.,* 2008).

If cells have already progressed through S-phase before DNA damage, cell cycle arrest and prevention of entry into mitosis is controlled through ATR activation of CHK1 which phosphorylates CDC25A, B and C to prevent activation of CDK1/cyclinB (Uto *et. al.,* 2004).

Many other targets for CHK1 and CHK2 exist, here I have given a brief summary of their functions, a more comprehensive explanation into their other functions can be found in Smith *et. al.* (2010).

1.3.1. Nuclear fallout and centrosome inactivation

The response of the syncytial *Drosophila* embryo to chromosomal abnormalities is to eliminate damaged nuclei from the embryonic cortex to deep inside the embryo, during cycles 10-13. Such yolk nuclei play no further role in development (Takada, Kelkar and Theurkauf, 2003; Sullivan, Fogarty and Theurkauf, 1993). Nuclear fallout involves co-ordinating multiple processes including centrosome inactivation and severing the connection between the embryonic cortex and MTs. Although the exact mechanism is unknown, it is thought that defects in DNA replication, or DNA damage during S phase, in

individual nuclei lead to the removal of the γ-TuSC proteins γ-tubulin, GRIP84 and GRIP91 from centrosomes at the onset of mitosis (Sibon *et. al.,* 2000). As the embryo continues to cycle through mitosis, the mitotic spindles that form around these nuclei do so acentrosomally, presumably from chromatin and augmin-derived MTs. Again, due to the syncytial nature of the embryo, it will continue to progress through to anaphase, even if it possesses some nuclei that have yet to align their chromosomes due to acentrosomal spindle inefficiency. Thus, those nuclei whose centrosomes have been inactivated have an increased chance of failure of chromosome segregation in anaphase. During the following interphase, such nuclei, which have lost their interactions between the nuclei, the centrosomes and the embryonic cortex, are actively transferred into the interior of the embryo.

How the embryo co-ordinates nuclear fall-out is currently unclear. The checkpoint kinase CHK2 (see Section 1.3.) appears to be involved. In *Drosophila*, a null mutation in the Chk2 homolog, Mnk , blocks centrosome inactivation, while DNA damage results in increased localisation of CHK2 to centrosomes (Takada, Kelkar and Theurkauf, 2003).

However, this linear pathway is only one that results in nuclear fallout. For example, defective spindles within the embryonic population, brought about by defective MAPs, will also lead to anaphase abnormalities and subsequent nuclear-fallout. It is therefore difficult to accurately assess whether nuclear-fallout in a specific perturbed background is due primarily to chromosomal or MT defects.

1.3.2. Histone variant 2A

Histone proteins form part of nucleosomes that make up chromatin. Each nucleosome contains a histone octamer consisting of two copies of each of the canonical histones (H2A, H2B, H3 and H4) (Luger *et. al.,* 1997). Histones are important for chromatin structure and function.

Histone variant 2A (H2AV), first described in 1980 (West and Bonner, 1980), is a variant histone protein involved in the DNA damage response (reviewed in Talbert and Henikoff, 2010). In comparison to non-variant histones which are synthesised during S-phase, variant histones are constitutively expressed at a basal level (Wu and Bonner, 1981). Upon double-strand DNA breaks H2AV rapidly becomes phosphorylated, creating local structural changes that allow DNA repair. It is phosphorylated at serine137, located in the SQ motif at it's C-terminus, forming γH2AV (Rogakou *et. al.,* 1998). γH2AV also mediates further recruitment of the MRN complex, through MDC1, which incorporates ATM to amplify the response (Stucki *et. al.,* 2005; Lukas *et. al.,* 2004; Uziel *et. al.,* 2003). More recently it has also been found that in HeLa cells CHK2 directly mediates the mitotic phosphorylation of H2AX (the human ortholog of H2AV) on the same C-terminal phosphorylation site (Tu et al. 2013).

1.4. Stem loop-binding protein

Stem loop-binding protein (SLBP) is a cell-cycle regulated protein that has an essential function in the regulation and processing of histone mRNAs.

Histone mRNAs are the only animal mRNAs to lack polyA tails, instead ending in a conserved 16nt stem loop. The history of the discovery of how histone mRNAs are processed is described in a review by Dominski and Marzluff

(1999). The lack of a polyA tail and presence of a conserved stem-loop structure was realised through cloning of histone genes in sea urchins (Sures , Lowry and Kedes, 1978; Cohn, Lowry and Kedes, 1976). This stem-loop was then found to be essential for 3' end processing by Birchmeier, Grosschedel and Birnstiel (1982). Initially the effector protein that binds the stem-loop was unknown (Vasserot, Schaufele and Birnstiel, 1989), and it was not until 1995 when Williams and Marzluff classified SLBP as the binding partner. SLBP binds to the 16nt stem loop and protects the mRNA before it can be translated, as well as mediating export from the nucleus and cleavage of the 3' end (Sullivan *et. al.,* 2001).

SLBP is also required for regulated expression of histone proteins with the cell-cycle. In humans this is achieved through cell-cycle regulation of levels of SLBP. Low levels of expression are present in G_1 and then a dramatic increase is seen as cells commit to S-phase (Dominski and Marzluff, 1999). In *Drosophila* SLBP levels are constant throughout the cell cycle (Lanzotti *et. al.,* 2004); therefore its sub-cellular localisation is the primary determinant of histone expression. In G_2 it becomes predominantly nuclear, to allow histone production in preparation for mitosis; on exit from mitosis into G_1 it becomes down-regulated (Lanzotti *et. al.,* 2004).

Loss of SLBP, via RNAi knockdown, has been found to result in decreased rates of DNA synthesis, accumulation of cells in S-phase, and retention of histone mRNAs in the nucleus in *Drosophila* U2OS cells (Sullivan *et. al.*, 2009). In addition, reduction in SLBP levels causes genomic instability: increased

chromosome breaks, loss of heterozygosity, tetraploidy and position effect variegation, in slbp null mutant *Drosophila* embryos (Salzler *et. al.,* 2009).

In humans, a deficiency in the Slbp locus is characteristic of the disorder known as Wolf-Hirschhorn Syndrome (WHS) (Kerzendorfer *et. al.,* 2012). Symptoms of this disease include mental retardation, seizures and delayed development. WHS is caused by a genetic deletion in the short arm of chromosome 4, where the Slbp gene is located. Kerzendorfer *et. al.* (2012) found that reduced SLBP levels cause cell cycle delay, impaired S-phase progression and impaired DNA replication. This paper describes WHS, for the first time, as a disorder of impaired chromatin organisation.

SLBP is not thought to bind any other 3'UTRs in the cell. A study by Townley-Tilson *et. al.* (2006) used recombinant-RIP-ChIP analysis to identify nucleic acids that were bound by human SLBP. Their study concluded that the replication-dependent histone mRNAs are the only mRNAs that SLBP associates with. Indeed, for the last twenty years, the exclusive role described for SLBP is the regulation of histone mRNA transport and translation.

1.5. Previous work

During my undergraduate research project I studied the potential role of SLBP as a mitotic MAP. A previous investigation by the Wakefield laboratory (unpublished) had identified multiple *Drosophila* proteins that likely had roles as MAPs during mitosis, one of these was SLBP. Live imaging of *Drosophila* embryos expressing a GFP-fusion to SLBP discovered a weak localisation to the area of the mitotic spindle (Supplementary materials). However biochemical

analysis found that SLBP did not pellet with microtubules during a microtubule co-sedimentation assay (Supplementary materials). The results of this biochemical analysis were not clear enough to be conclusive; therefore the question of whether SLBP is a mitotic MAP was left in doubt.

In addition, a mass spectrometry analysis had been performed on a sample of GFP-TRAP-A beads that had been incubated with *Drosophila* extract of embryos expressing SLBP-GFP. Following removal of any false positives and proteins below a standard threshold used routinely in the laboratory, a large number of SLBP interactor proteins were identified. These ~130 proteins fell into two classes - proteins with roles in mRNA processing and translation (e.g. ribosomal proteins, translation factors, splicing factors), and other proteins. Given the known role of SLBP in regulating histone mRNA processing and translation, I focused on the other 32 proteins. Using standard Flybase Gene Ontology (GO) searches, the majority of these interactors fell into three main categories: chromosome cohesion, functions at the centrosomes, and functions at the embryonic cortex (Table 1-1). One interpretation of these results is that SLBP may be performing additional functions to its known role in histone mRNA processing. This thesis sets out to explore this hypothesis.

CORTEX	CENTROSOMES	CHROMOSOME COHESION	OTHER
Spg	AurA	SMC1	GTP1/CG10628
Ced-12/ELMO	Asl	SMC2	Beg
Sgg	Sas-6	SMC3/CAP	Tao
CKIε	Grip84	SMC4	CG10672
AP-2μ	Grip91	NippedB/SCC2	CG9331
AP1-2-β		SCC3	CKIIβ
Muskelin		Top2	CG31688
Sec15			CG12702
CG6617			Larp
			East
			Gnf1

Table 1-1. Identified SLBP-interactor proteins. Mass spectrometry analysis on a sample of GFP-TrapA beads used to immuno-precipitate SLBP-GFP and bound interactors from embryo extract. Results were filtered to remove false positives, anything with a score <30, <3 peptides and any proteins with functions in translation or part of the ribosomes. 32 proteins remained which can be grouped into having functions at the cortex, the centrosomes, in chromosome cohesion or other.

1.6. Masters by Research project

In this project I aimed to determine whether SLBP does work as a mitotic MAP and to discover any potential further functions it carries out.

To assess mitotic MAP potential, I repeated the microtubule co-sedimentation assay from my undergraduate project with metaphase-arrested embryos, to obtain a clear biochemical result of whether SLBP associates with MTs in a mitotic sample.

To assess potential further functions SLBP may possess, I utilised multiple approaches. RNA-Seq analysis was performed to identify SLBP's association with interactor protein mRNAs, and to clarify if it acts upon proteins or mRNAs. This was also investigated by protein level and localisation studies in an RNAi line. Characterisation of the RNAi phenotype was also carried out; as well as staining with an antibody that recognises DNA damage, to support that results were not a consequence of the DNA damage response.

2. Materials and Methods

2.1. Fly husbandry

2.1.1. Fly maintenance

Flies were maintained in standard vials or bottles (Dutscher Scientific) on standard culture media (see supplementary information). Transfer to fresh vials/bottles was carried out as necessary, and addition of de-ionised water and dried yeast was carried out as seen fit, to encourage larval growth. All lines were maintained at 25°C. Some lines were kept at 18°C or 28°C periodically, to delay/speed up development, respectively. Occasionally flies were kept on food containing 0.006% tetracycline (GERBU) to eradicate Wolbachia infection.

2.1.2. Fly lines

All lines were obtained from Bloomington Stock Centre, unless otherwise stated.

Histone-RFP*UASp-EB1-GFP/Cyo ; Maternal-α-Tubulin-VP16-Gal4/MKRS (Wakefield laboratory)

$slbp^{10}$/$TM3GFP^{SbSerr}$ on 3rd

$slbp^{EP1045}$/$Tm6b^{Tb}$ on 3rd

w67 (control line)

pTRiP-Slbp-Valium20 on 2nd (Harvard Medical School)

UASp-GFP-Slbp on 3rd (Iampietro *et. al.*, 2014)

Maternal-α-Tubulin-VP16-Gal4 on 3rd

2.1.3. Genetic crosses

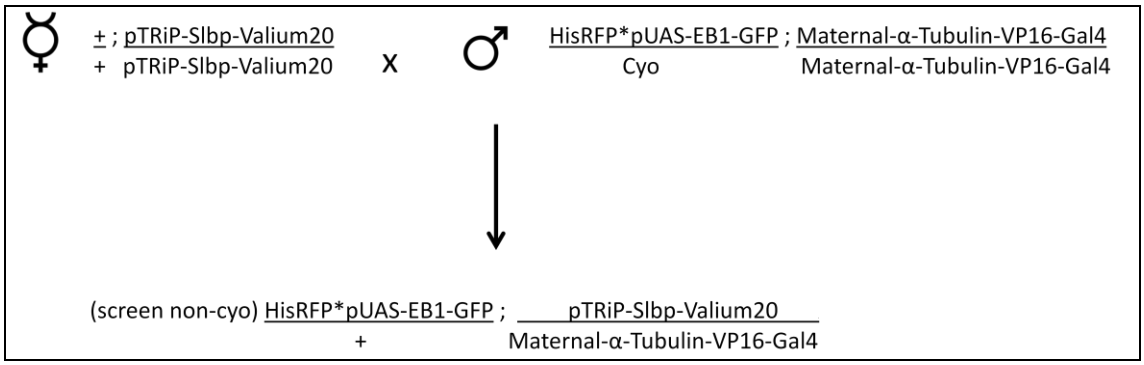


Figure 2-1. Cross set up to produce flies expressing RNAi for SLBP and copies of HistoneRFP and EB1GFP for imaging and collections. Homozygous virgin females expressing pTRiP-Slbp-Valium20 were selected to breed with males expressing Histone-RFP, EB1-GFP, and Maternal-α-Tubulin-VP16-Gal4. Non-curly winged individuals were selected from the resulting progeny to ensure they had all the desired transgenes.

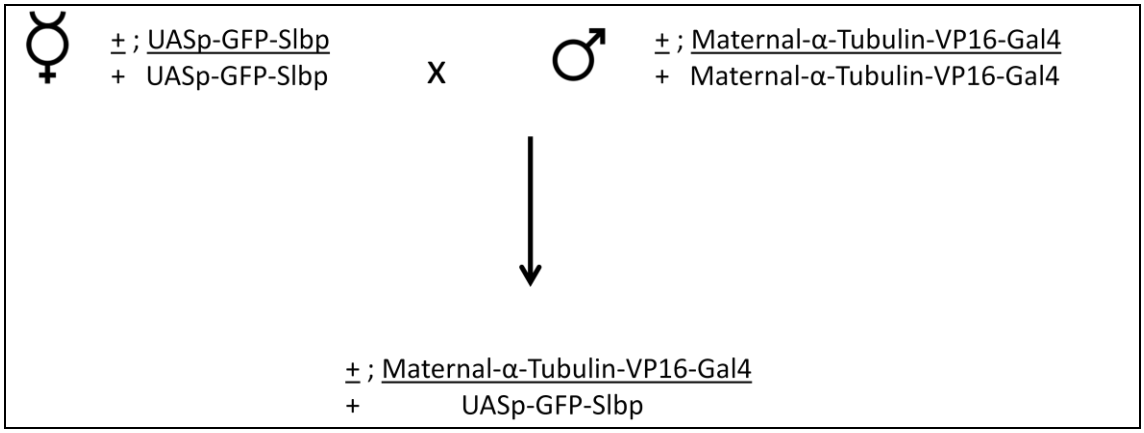


Figure 2-2. Cross set up to produce flies expressing an SLBP-GFP construct for MT co-sedimentation analysis. Homozygous virgin females expressing a GFP-Slbp construct were crossed with males homozygous for the version of the UASp driver, Gal4, which is expressed with maternal α-tubulin. Offspring possessed a copy of each gene, allowing expression of the GFP construct in embryos.

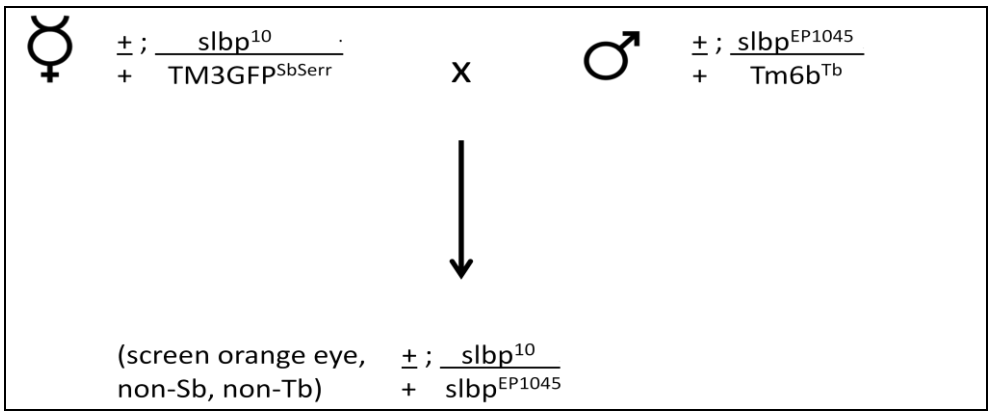


Figure 2-3. Cross set up to produce flies transheterozygous for mutations in SLBP. Heterozygous slbp mutant lines were crossed to produce offspring with a copy of each mutation. Offspring were screened for orange eyes, non-stubble hairs and non-tubby phenotype and were viable sterile.

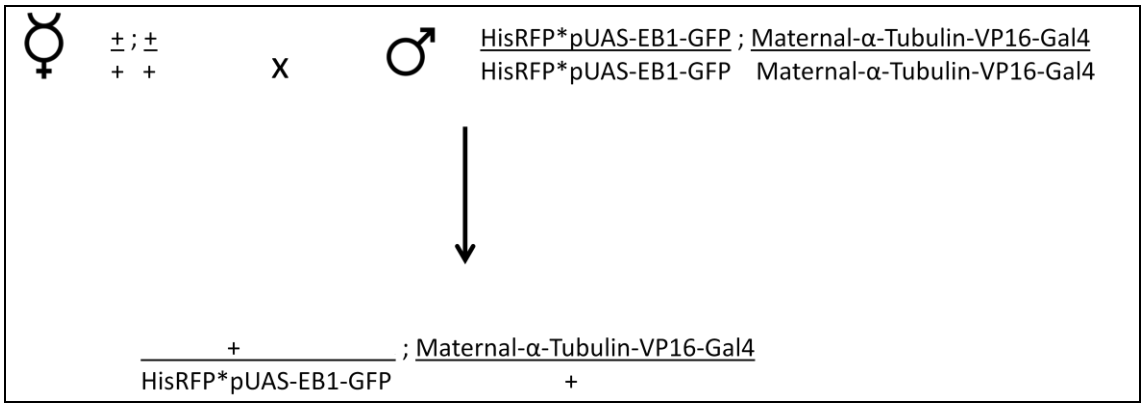


Figure 2-4. Cross set up to produce heterozygous flies expressing HistoneRFP and EB1GFP for hatch rates. Homozygous males with transgenes Histone-RFP, EB1-GFP, and Maternal-α-Tubulin-VP16-Gal4 were crossed with w67 virgin females. The resulting progeny were used for hatch rate analysis.

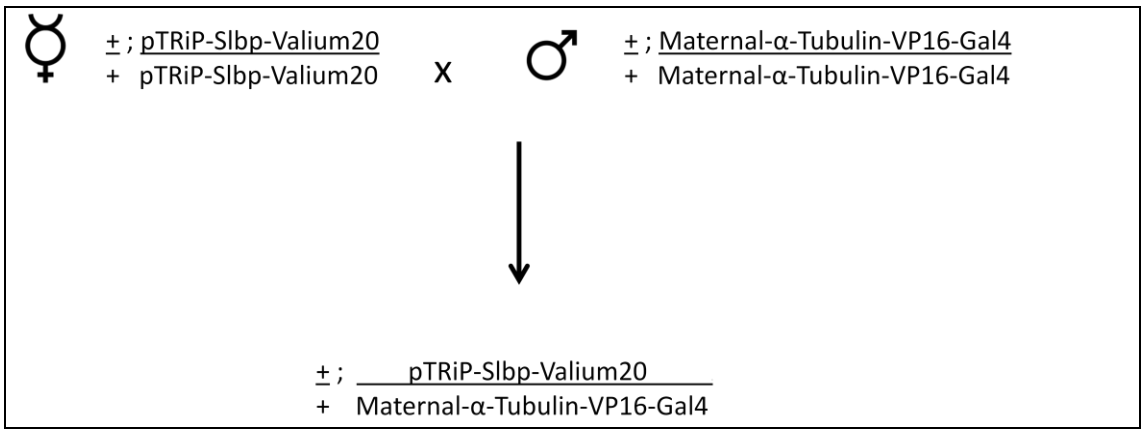


Figure 2-5. Cross set up to produce flies expressing RNAi for SLBP, without HistoneRFP and EB1GFP transgenes, for hatch rates. Homozygous virgin females expressing pTRiP-Slbp-Valium20 were selected to breed with males expressing Maternal-α-Tubulin-VP16-Gal4 to produce offspring which would lay embryos with an RNAi knockdown of Slbp, without co-expressing Histone-RFP and EB1-GFP.

2.1.4. Embryo collection and treatment

Embryo collection:

Flies were placed in embryo collection chambers, sealed at one end with apple juice agar plates (2.5% Agar (Lab M Ltd.), 27.5% apple juice, 70% dH_2O). Plates were changed at 3h intervals and washed in bleach to de-chorionate embryos. Embryos were then washed through a vacuum filter (Millipore) with 0.1% Triton (Sigma), collected in 1.5ml plastic tubes (Eppendorf) and flash-frozen in liquid nitrogen.

MG132-treatment:

To arrest them in mitosis before freezing, after collection and bleach de-chorionation as above, embryos were incubated in a solution containing 66.5% PBS (Melford), 33.2% heptane (Sigma) and 0.3% MG132 (Sigma) for 20mins

and then washed with 0.1% Triton (Sigma) through a vacuum filter (Millipore). Control embryos were untreated.

Fixing *Drosophila* embryos:

Embryos were collected and bleach de-chorionated as above. Following this, embryos were fixed in a 1:1 solution of methanol (Fisher) and heptane (Sigma) for 30s, and re-suspended in methanol. Fixed embryos were stored at 4°C until needed.

2.2. Imaging

Manual de-chorionation:

One to two hour old embryos were collected from chambers and manually de-chorionated as described in Hayward *et. al.* (2014).

2.2.1. Live imaging

One to two hour old, manually de-chorionated embryos were visually screened for those in cycles 9-12 and then imaged using a Visitron Systems Olympus IX81 microscope with a CSO-X1 spinning disk. Z-stacks, consisting of 5 x 1µm steps, were captured at 10sec intervals under a 60x oil objective, NA=1.4 (Olympus). This was carried out at room temperature. Processing was performed using Fiji/ImageJ software (See Section 2.2.4.).

2.2.2. Immuno-staining

Zero to three hour old methanol-fixed embryos were re-hydrated in 3 x 10min washes in PBST (PBS-Melford, 0.001% Tween-Fisher), then blocked for 30min in a 3% BSA (Sigma) in PBST solution. Following this embryos were incubated with primary antibodies in PBST (see section 2.3.6.) on a rotatory shaker at 4°C

overnight, washed 3 x 10min in PBST, and incubated for 2h at room temperature with the secondary antibodies in 3% BSA (see section 2.3.6.). Three final PBST washes were performed, the second wash containing Hoechst 33342 (1:1000 dilution) (Invitrogen), before embryos were mounted on a standard microscope slide in mounting media (2% N-N-Propylgallate (Sigma), 85% glycerol (Fisher), 13% H_2O), sealed with a coverslip and clear nail polish.

2.2.3. Fixed imaging

Fixed and stained embryos were imaged using a Zeiss 510 Axiovert 200M Inverted Meta confocal microscope with a 63x oil objective. Images were processed using Fiji/ImageJ software (See section 2.2.4.).

2.2.4. Image processing

Image processing was perfomed using Fiji/ImageJ software. For creation of live movies, z-stack images were concatenated and maximum projections created. Background levels were adjusted to improve image quality and scale bars added.

For fixed images, maximum projections were created from 1μm slice stacks and signal intensities measured. Firstly, line plots were created by manually drawing a line through individual spindles and then measuring signal intensity along that line. Secondly, using the circle tool, all centrosomes in images were manually selected and signal intensity quantified. The same size and shape circle was then used to select sites directly between spindles to quantify background cytoplasmic signal intensity.

2.3. Biochemistry

2.3.1. RNA-Seq

A sample of GFP-TRAP-A beads (Chromotek) that were incubated with an extract of SLBP-GFP-expressing embryos to precipitate SLBP, and bound interactors, from my undergraduate project, was sent to the Exeter Sequencing Service for RNA-Seq analysis.

2.3.2. Gel electrophoresis

Both control and MG132-treated frozen embryos were prepared by homogenization in 1x sample buffer (88% PSB (TrisHCL pH6.8 (BioRad), SDS (Melford), Glycerol (Fisher), M-β-mercaptoethanol (Sigma), EDTA (Sigma) and Bromophenol blue (Sigma)): 12% Dithiothreitol (Sigma)), and boiling in a heat block (~95°C) for 5-10 mins. Samples were then loaded onto a 10% or 15% SDS-polyacrylamide gel (depending on the expected size of the protein of interest; anything below 30kDa was run on a 15% gel) and run at 150v for approx. 90mins.

2.3.3. Coomassie stain

To stain for total protein, gels were stained with GelCode™ Blue Safe Protein Stain (ThermoScientific) until bands appeared, then de-stained in distilled water until background levels were reduced to an acceptable level.

2.3.4. Western blotting

For Western blot analysis, gels were removed and transferred onto nitrocellulose membranes (ThermoScientific) for 1h at 250mA. The membranes were blocked in 5% milk in PBST (blocking buffer) for 1h at room temperature, on a 3D rocking platform. Then the membranes were incubated with primary

antibody in blocking buffer (see Section 2.3.6.) at 4°C overnight on a rotatory shaker. Following 3 x 5min washes in PBST, membranes were incubated with secondary antibody in blocking buffer (see Section 2.3.6.) for 90min at room temperature on a 3D rocking platform. Finally membranes were washed 3 x 5min each in PBST before being exposed onto photofilm via Enzyme Linked Chemiluminescence (ECL) as described in the manufacturer's instructions (BioRad).

2.3.5. Microtubule co-sedimentation assay

0.1g of frozen embryos, expressing GFP-tagged SLBP, were homogenised and a MT co-sedimentation assay performed, as described in Hughes *et al.* (2008). Samples of the final extracts (-taxol supernatant (SN), -taxol pellet (P), +taxol SN and +taxol P) were subjected to gel electrophoresis and Western blotting, as described above.

2.3.6. Antibody list

Dilution for all secondary antibodies in Western blots was 1:10,000, and for all secondary antibodies in stainings was 1:1000.

Antibody	Source	Dilution for western blots (if used)	Dilution for staining (if used)
Primary antibodies:			
DM1A	Invitrogen	1:5000	1:1000
Rabbit anti-gamma-tubulin	Invitrogen	1:1000	1:1000

Rabbit anti-AurA	Invitrogen	1:1000	1:1000
Mouse anti-gamma-tubulin	Invitrogen	-	1:1000
Rabbit anti-Grip91	Oegema *et. al.* (1999)	1:1000	1:250
Rabbit anti-Grip84	Oegema *et. al.* (1999)	1:1000	1:250
Guinea-pig anti-SLBP	Iampietro *et. al.* (2014)	1:1000	-
Mouse anti-Actin	Invitrogen	1:1000	-
Rabbit anti-HisH3	AbCam	1:1000	-
Rabbit anti-HisH2A	Millipore	1:1000	-
Mouse anti-γH2AV	Deposited to the DSHB by Hawley, R.S. (DSHB Hybridoma Product UNC93-5.2.1)	1:1000	1:200
Mouse anti-GFP	Invitrogen	1:1000	-
Secondary antibodies:			
Anti-mouse Alexa 488	Invitrogen		
Anti-rabbit Alexa	Invitrogen		

488			
Anti-rabbit Alexa 555	Invitrogen		
Anti-rabbit Alexa 633	Invitrogen		
Anti-mouse Alexa 633	Invitrogen		
Anti-mouse HRP	Invitrogen		
Anti-rabbit HRP	Invitrogen		
Anti-guinea-pig HRP	Invitrogen		

Table 2-1. List of antibodies used in experiments.

2.4. CHK2 consensus sites screen

SLBP-interactor and control FASTA protein sequences were scanned, using ScanProsite (De Castro *et. al.,* 2006), for the CHK2 phosphorylation consensus site described in Seo *et. al.* (2003).

B^{α} – X – R – X – X – S/T

B^{α} – hydrophobic amino acid

Control proteins were selected from cluster6 *Drosophila* proteins with mRNA expression characteristics similar to Slbp (Roy *et. al.,* 2010). An online random number generator was used to select 32 proteins, from cluster 6, and then these were sorted for a group with a similar molecular weight profile as our SLBP-interactors (to ~20kDa), proteins outside this distribution were removed

and more proteins randomly selected and screened until 32 proteins had been selected.

3. Results

3.1. SLBP-GFP does not associate with microtubules during mitosis

SLBP was identified by proteomics analysis as a putative mitotic MT associated protein (Wakefield laboratory, unpublished). During my undergraduate research project I attempted to assess this behaviour, through investigating the dynamic localisation of a GFP-SLBP fusion protein expressed in *Drosophila* early embryos. That study demonstrated that SLBP-GFP localised weakly to the area of the mitotic spindle in early *Drosophila* embryos, via live confocal fluorescence imaging (Supplementary materials). A preliminary biochemical analysis, performing a MT co-sedimentation assay, was inconclusive, possibly due to the use of cycling embryos, a large proportion of which would be in stages outside of mitosis (Supplementary materials). One of the aims of this Masters project was therefore to clarify these preliminary results, through repeating the MT co-sedimentation assay with mitosis-arrested embryos.

To assess whether SLBP-GFP biochemically associates with MTs in mitotic embryos, 0-3 h embryos expressing SLBP-GFP were treated with the drug MG132, which prevents the metaphase-anaphase transition through inhibition of the 26S proteasome (Genschik *et. al.,* 1998), during the collection process. Extracts of these embryos were then subjected to a MT co-sedimentation assay, the samples obtained run on a gel and a western blot carried out. The predicted moleclular weight for SLBP is 31kDa and for GFP is 27kDa, giving a total predicated molecular weight of 58kDa. However, SLBP-GFP in this blot appears to be about 70kDa. Previous work has found that SLBP has unusual dynamics on an SDS polyacrylamide gel that cause it to run at about 45kDa (Dominksi and Marzluff, 1999), which is also what I observed in my western blot

for SLBP (Fig. 3-3). With a GFP tag the resulting observed molecular weight would be 72kDa. Results clearly showed that a single band of ~70kDa was identified from extracts expressing SLBP-GFP, and that this band does not bind MTs (Fig. 3-1). Samples with taxol added to them form cold-stable MTs which pellet (P) when centrifuged at high speed, whereas samples without taxol do not form stable MTs and tubulin subunits should remain in the supernatant (SN). Clear bands for α-tubulin in the -taxol SN and the +taxol P demonstrate that tubulin successfully pelleted only when expected. However, no anti-GFP signal was present in the +taxol P, showing that SLBP-GFP does not pellet with MTs and therefore does not biochemically associate.

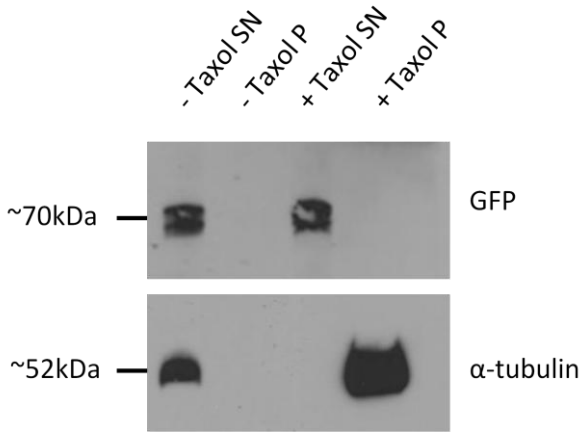


Figure 3-1. SLBP-GFP does not associate with microtubules during mitosis. Western blot of the samples collected during a MT co-sedimentation analysis experiment of metaphase arrested SLBP-GFP-expressing embryos, probed for GFP and α-tubulin. α-tubulin shows that the MT co-sedimentation assay was successful as tubulin pelleted in the +taxol sample and did not in the -taxol sample (remaining in the supernatant (SN)), taxol induces microtubule polymerisation. However, no GFP was detected in the +taxol pellet (+taxol P) so SLBP did not pellet with microtubules.

3.2. SLBP-GFP interacts with a set of cellular proteins enriched for specific gene ontologies, but does not do so through interacting with the mRNAs of these interacting proteins

During our previous preliminary investigations, an immuno-precipitation (IP) and mass spectrometry (MS) analysis identified a large number of GFP-SLBP-interacting proteins (E. Anderson, Final year project, University of Exeter). After removal of proteins which could be directly related to the sole known function of SLBP, in regulating processing and translation of histone mRNAs, 32 interactors remained which could be grouped into three functional ontologies: (i) centrosome function, (ii) chromosome cohesion and (iii) embryonic cortex functions (Table 1-1). This MRes project began by considering why SLBP might immuno-precipitate these sets of proteins. A previous study in human tissue culture cells, using a chromatin IP approach concluded that the replication-dependent histone mRNAs are the only mRNAs that SLBP binds (Townley-Tilson *et. al.,* 2006). However, it is possible that *Drosophila* SLBP in embryos binds its interacting proteins indirectly, as a consequence of binding their mRNAs, during the process of translation. This hypothesis was deemed possible as the second highest scoring protein in the mass spectrometry data was La motif-related protein (Larp), whose function is to regulate a subset of proteins that contain a 5'terminal oligopyrimidine tract (5'TOP) (Aoki *et. al.,* 2013). The 5'TOP is a common feature observed in all sequenced vertebrate ribosomal protein mRNAs. It usually consists of a cytidine residue at the cap site, followed by 7-13 pyrimidine nucleotides, and has been found to be involved in the translational control of ribosomal mRNAs (Levy et al. 1991).

Given the known function of SLBP in binding the 3'end of histone pre-mRNA (Sullivan *et. al.,* 2001), I hypothesised that SLBP and Larp may work in

conjunction to regulate the subset of mRNAs encoding the proteins identified via MS. I undertook RNA-Seq analysis of SLBP-GFP IPs, assessing if the mRNAs of SLBP-interactors were enriched compared to a control sample. Approximately 0.4g of SLBP-GFP expressing, 0-3 h embryos were subjected to standard GFP-TRAP-A immuno-precipitation. Total RNA was extracted from the washed GFP-TRAP-A beads and RNA-Seq and analysis performed (Exeter Sequencing Service).

Each RNA sequence was mapped to the corresponding location within the *Drosophila* genome (www.flybase.org (Flybase)) and assigned an abundance value (RPKM - Reads Per Kilobase per Million). It was then compared with existing high-throughput expression data for 0-2 h and 2-4 h embryos (Flybase). If SLBP interacts with the mRNAs of the proteins identified via MS, we would expect the abundance of these specific mRNAs to be increased in samples extracted from SLBP-GFP expressing embryos, in relation to the total mRNA value as assigned by the Flybase high throughput data. However, analysis of the RNA-seq results demonstrate that the abundance of mRNAs in the SLBP-GFP sample generally correspond equally with the Flybase 0-2 h or 2-4 h expression values. For example, a visual comparison of a region of Chromosome 2L (Figure 3-2. A), shows the abundance of mRNAs between SLBP-GFP and Flybase expression data to be similar. However, one highlighted region was found to have a high abundance in the SLBP-GFP RNA-seq, while not being present in the Flybase early embryo expression data. Upon closer analysis, this region was found to correspond to the histone genes. This is to be expected, given that the Flybase expression data is generated using an oligo-T primed approach - histone mRNAs are the only mRNAs that do not have

a polyA tail (Marzluff, 2005) and therefore do not have an expression value via the Flybase high-throughput data.

Graphical representations comparing the abundance of mRNAs of SLBP-interactors versus the normal expression levels (FlyBase) verify the visual analysis (Figure 3-2. B and C) - the mRNAs of the proteins identified as interacting with SLBP-GFP are not enriched in the IP, in comparison to total mRNA present in the embryo. Both 0-2h and 2-4h control samples were tested to encompass all stages in the 0-3h SLBP sample. These results suggest that, rather than interacting with a specific subset of mRNAs through Larp, or independently of Larp, the interaction between SLBP-GFP and the other proteins occurs through protein:protein interactions.

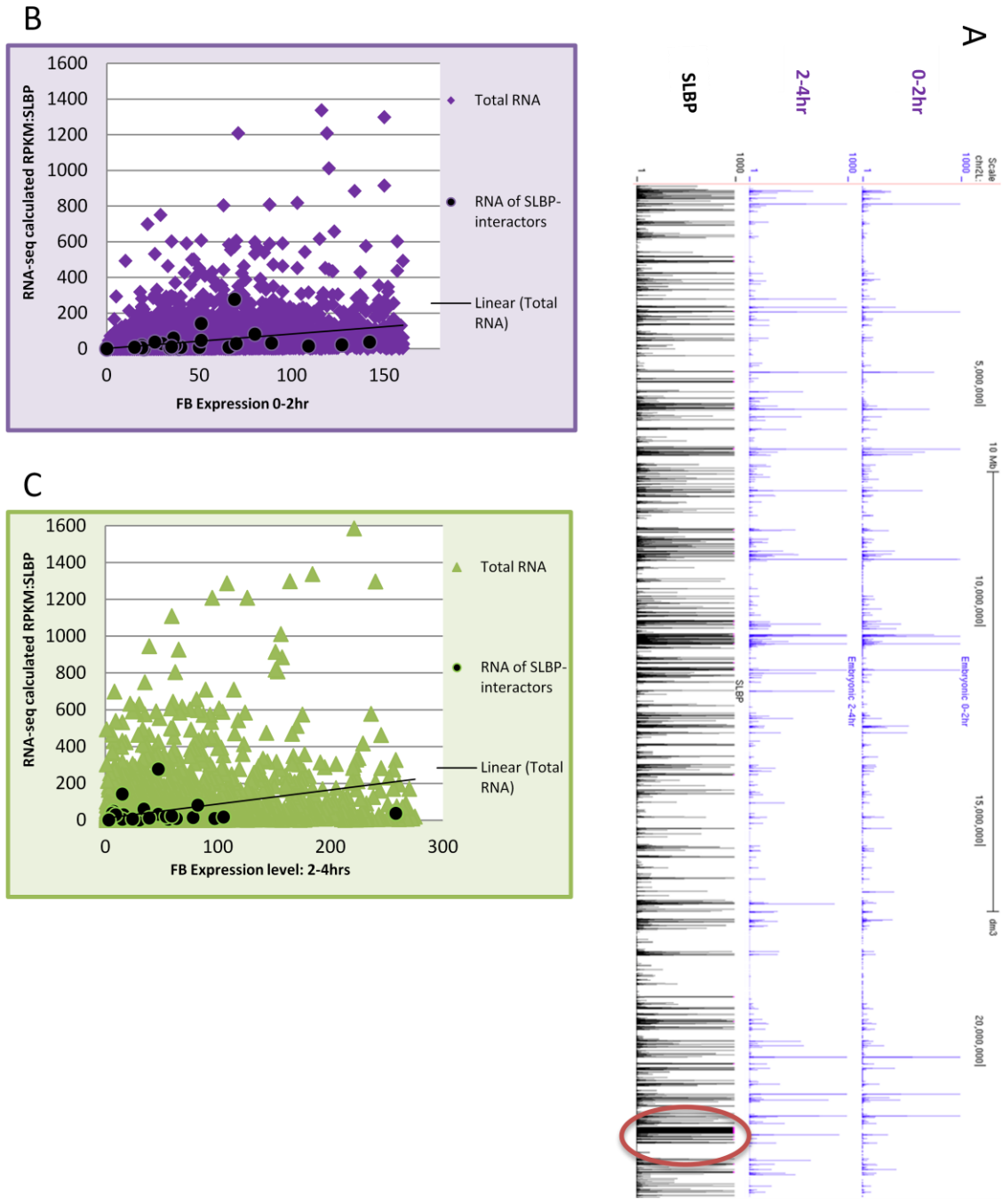


Figure 3-2. SLBP does not appear to associate with the mRNAs of interacting proteins.

(A) RNA-Seq analysis of control whole embryo 0-2h and 2-4h embryo extract and sample from immuno-precipitation of GFP from 0-3hr SLBP-GFP-expressing embryos. RNA levels do not appear increased in the SLBP extract except for the highlighted region which represents the histone RNAs. (B) Graphical representation of RNA-Seq data highlights that SLBP-interactor protein RNAs (black dots) are not increased in the SLBP sample with respect to 0-2h whole embryo extract. FB expression = Normal expression levels, taken from information on www.flybase.org (C) Graphical representation of RNA-Seq data highlights that SLBP-interactor protein RNAs are not increased in the SLBP sample with respect to 2-4h whole embryo extract.

3.3. Reducing SLBP levels in the early embryo results in multiple mitotic phenotypes

A previous fixed analysis of slbp mutant embryos described multiple cell-cycle related phenotypes, including nuclear fallout, chromatin abnormalities and spindle defects (Sullivan *et. al.,* 2001). In this study, the authors conclude that the defects seen upon Slbp disruption can be explained solely by the role of Slbp in regulating histone translation. Their hypothesis is that loss of SLBP leads to reduced histone levels and reduced incorporation of histones into replicating chromatin. This results in DNA damage and chromatin breakage - which results in spindle abnormalities, chromosome segregation defects and ultimately loss of resultant nuclei from the embryonic cortex, through nuclear fallout, as a consequence of the DNA damage response.

However, given the interaction between SLBP-GFP and proteins with functional ontologies that are related to these reported phenotypes, I therefore wondered whether SLBP has additional, undiscovered roles in the early embryo relating to regulating the function of these classes of proteins. To begin to investigate this, I undertook a detailed live analysis of mitosis in embryos in which SLBP had been knocked down using RNAi. A fly line carrying a GAL4-inducible shRNA specific for the Slbp gene (pTRiP-Slbp-Valium20) was obtained from Harvard Medical School and crossed to a line expressing GAL4 in the female germline, in order to reduce levels of SLBP in the early embryo via maternally reduced synthesis in oogenesis, henceforth slbp-RNAi flies (Figure 2-1). Western blotting of control and slbp-RNAi embryos using an anti-SLBP antibody (Iampietro *et. al.,* 2014) confirmed that a band corresponding to the size of SLBP was absent in the slbp-RNAi embryos (Figure 3-3).

Live imaging of *Drosophila* embryos expressing slbp-RNAi concomitantly with transgenes for EB1-GFP and Histone-RFP was carried out using a confocal spinning disc microscope, taking z-stack movies. A control line expressing only EB1-GFP and Histone-RFP was also imaged. EB1 is a protein that localises to the plus ends of growing MTs and is essential for proper MT assembly and function (Rogers *et. al.,* 2002).

Embryos were followed through cycles of mitosis and the organisation of centrosomes, microtubules and chromatin investigated. Multiple defects were observed, including centrosomes that did not nucleate astral arrays or contribute to spindle formation (centrosome inactivation), nuclei falling away from the cortex and into the yolk (nuclear fallout) and spindles forming in the absence of centrosomes (acentrosomal spindle assembly) (Figure 3-4 and Supplementary materials). The timing of these abnormalities was not consistent between embryos, with some embryos arresting at metaphase prior to these defects, and some embryos continuing to attempt mitosis in the presence of defects, until a terminal phenotype was achieved (data not shown). Only 10% of embryos developed to the larval stage (Supplementary Materials).

A reduction in SLBP levels in the early embryo therefore results in multiple mitotic defects that include centrosome inactivation, chromosome breakage / loss of cohesion and alignment, and nuclear fallout. Although these live phenotypes correlate well with the previous fixed analysis of slbp mutant embryos (Sullivan *et. al.,* 2001), and histone levels are reduced in slbp-RNAi embryos (Figure 3-5), they are not easily explainable solely on the basis of SLBP functioning to regulate histone levels. For example, in some instances,

slbp-RNAi embryos presented apparently normal chromatin, where condensation and alignment proceeded apparently regularly, but mitotic spindle formation was perturbed. Moreover, work by Günesdogan, Jäckle and Herzig (2014) showed that *Drosophila* histone null mutants had prolonged S-phases, arresting at G2 without accumulating DNA damage - a very different phenotype to that observed in slbp-RNAi embryos.

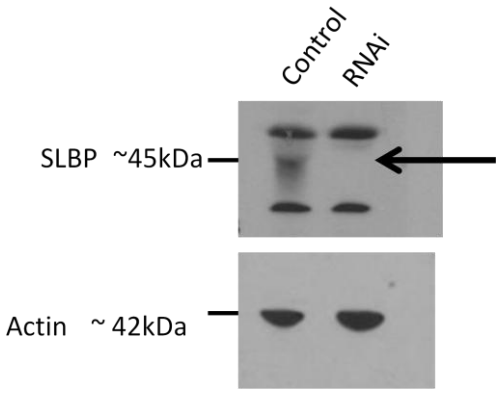


Figure 3-3. SLBP levels are reduced in slbp-RNAi embryos. Western blot of control and slbp-RNAi 0-3h embryo extracts. Arrow indicates expected molecular weight of SLBP which is absent in the RNAi-slbp lane. Higher and lower molecular weight bands surround SLBP, representing non-specific binding (Lecuyer, personal communication – we were informed that although the band corresponding to SLBP is diffused, this is how it was observed when it was created and does indeed represent SLBP. It is also of the correct molecular weight). (actin loading control)

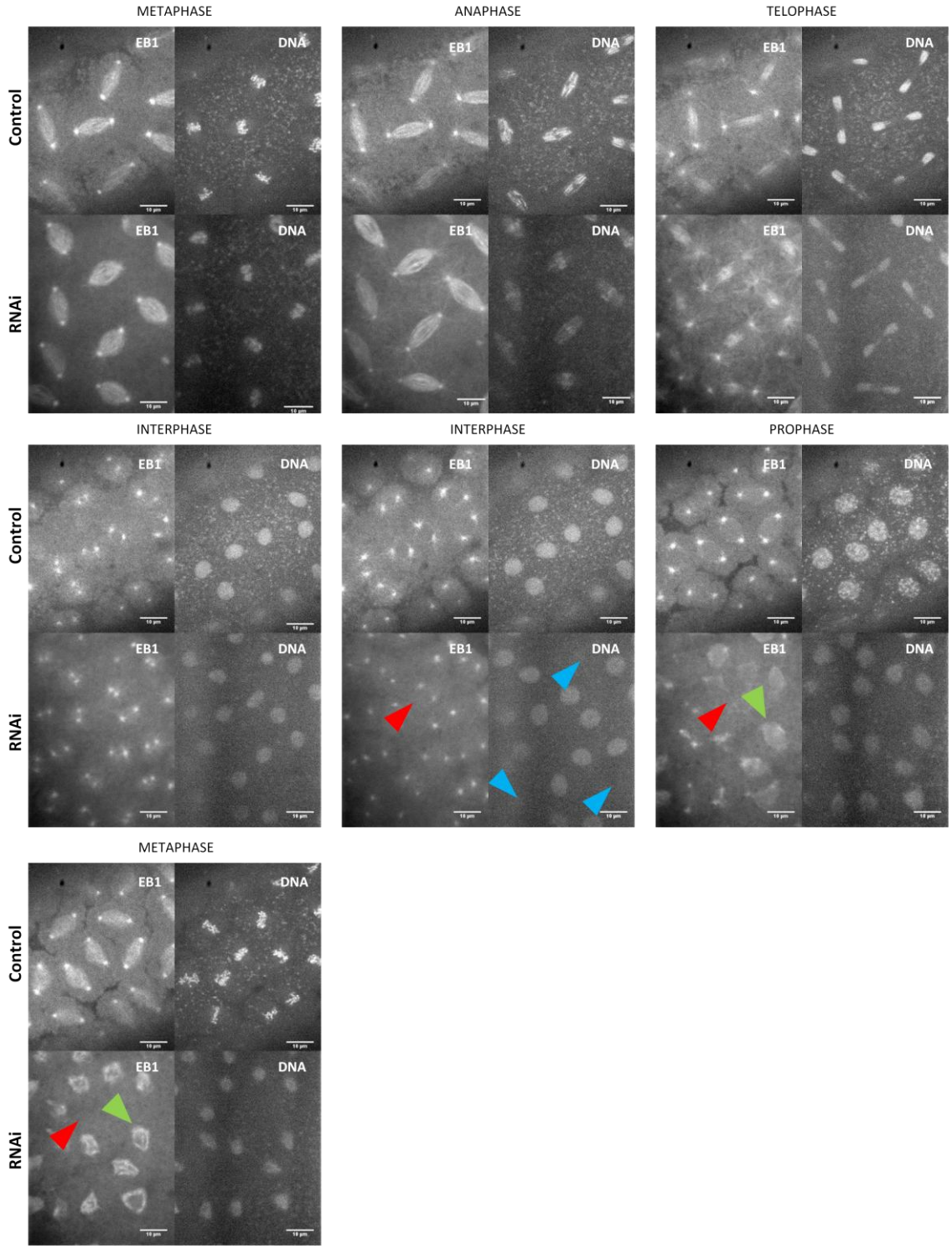


Figure 3-4. RNAi knockdown of SLBP causes multiple mitotic defects. Still images taken from z-stack projection movies, obtained on a spinning disk confocal microscope, of control and slbp-RNAi embryos co-expressing transgenes for EB1-GFP and His-RFP. Red arrows=inactive centrosomes, Blue arrows=areas of nuclear fallout, Green arrows=acentrosomal spindle assembly. (Full movies available in Supplementary Materials)

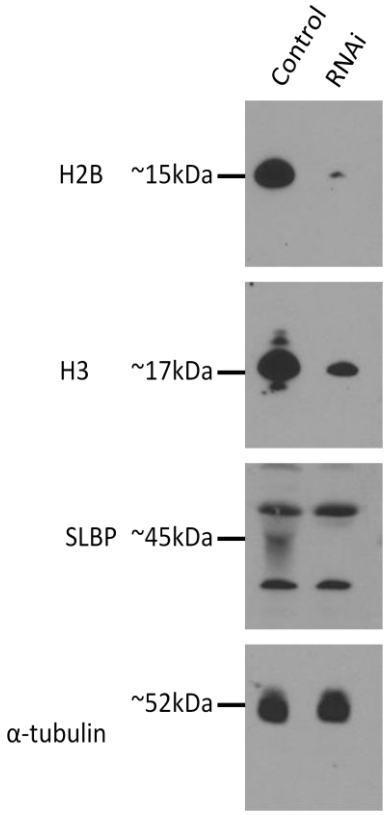


Figure 3-5. Histone levels are reduced in slbp-RNAi embryos. Western blot of 0-3h control and slbp-RNAi embryos. Levels of HistoneH2B and HistoneH3 are reduced in RNAi. (SLBP levels are reduced in the RNAi line, α-tubulin loading control)

3.4. Loss of SLBP does not alter the protein levels of centrosomal SLBP-interacting proteins, but does affect their cell-cycle dependent localisation

To further investigate the relationship between SLBP, its putative interacting proteins and the consequences of reducing SLBP function in the embryo, I focused on a subset of SLBP interactors within the "centrosomal function" ontology: Aurora A (AurA), Grip84 and Grip91. Time limitations precluded similar studies on other sets of SLBP interactors. Aurora A is a mitotic kinase that localises to centrosomes during mitosis, where it is responsible for phosphorylating target proteins with roles in microtubule nucleation (Terada, Uetake and Kuriyama, 2003). Grip84 and Grip91 (reviewed in Kollman *et. al.,*

2011) localise predominantly to centrosomes, and weakly to the mitotic spindle, in embryos (Colombié *et. al.,* 2006; Barbosa *et. al.,* 2000) and, together with γ-Tubulin, constitute the MT nucleating complex, the γ-TuSC.

First, I assessed the amount of Aurora A, Grip84 and Grip91 proteins present in control vs. slbp mutant ($slbp^{10}/slbp^{EP1045}$) or slbp-RNAi embryos via western blot analysis. The levels of three SLBP-interacting proteins remained the same in slbp mutant or slbp-RNAi lines compared to control (γ-tub internal control, α-tubulin loading control) (Fig. 3-5). This further supports the notion that SLBP is not necessary for the proper translation of these proteins, and does not have a role with them prior to protein formation.

I next sought to determine whether loss of SLBP in the embryo affected the cell-cycle dependent localisation of these interacting proteins. slbp-RNAi , or control embryos were collected, fixed, and stained using antibodies against γ-tubulin (γ-tub), Grip84 or Grip91, in addition to α-tubulin and DNA (Hoechst 33342) and imaged with a confocal fluorescence microscope. The intensity of γ-tub, Grip84 and Grip91 was quantitatively analysed using Fiji/ImageJ software. To determine whether the amount of each subunit at centrosomes was altered in slbp-RNAi embryos, the intensity of the corresponding fluorescence channel at centrosomes at metaphase was analysed in relation to cytoplasmic intensity, to control for background levels, and represented as bar charts. In addition, intensity line plots through the length of individual mitotic spindles were compared.

Line plots highlight clearly that, although some centrosomal Grip84 and γ-tubulin remains in slbp-RNAi embryos, the signal intensities at the centrosomes for these proteins are reduced in the RNAi line compared to control (Fig. 3-6.1 and Fig. 3-6.2). Moreover, although loss of centrosomal intensity for Grip91 in *slbp*-RNAi embryos is not clearly demonstrated via line plots, due to the very low signal this antibody generates, (Fig. 3-6.3), the bar charts generated from the centrosomal/cytoplasmic signal intensity data, shows that for all three proteins, the intensity at the centrosomes is significantly reduced from control levels (t-test $p<0.01$) in the *slbp*-RNAi embryos (Fig. 3-6.4). This data strongly suggests that SLBP is involved in the proper recruitment of these γ-TuSC proteins to the centrosomes.

A similar analysis was carried out for Aurora A. Imaging clearly showed a reduced intensity of AurA signal at the centrosomes in the RNAi line and line plots corroborate this visual assessment (Fig. 3-7, A and B). The graph depicted in Fig. 3-7 (C), of measured signal intensities at centrosomes and in the cytoplasm, highlights this further, with the intensity significantly lower at the centrosomes in the RNAi line (t-test $p<0.01$).

Together, the phenotypic data presented above demonstrates that loss of SLBP function in the early embryo results in loss of accumulation of the identified SLBP-interacting proteins, Aurora A, Grip84 and Grip91 to centrosomes during mitosis. It also seems likely that the inability to recruit these proteins is related to the centrosome inactivation phenotype described in Section 3.3. Previous studies have shown that centrosome inactivation is a regulated process in the

early embryo and that loss of MT nucleation correlates with a specific loss of Grip84 and Grip91 from the centrosome (Sibon *et. al.,* 2000).

It is possible that the loss of these centrosomal proteins and the centrosome inactivation seen upon loss of SLBP can be explained by the sole known role of SLBP in regulating histone processing and translation. Centrosome inactivation is triggered upon DNA damage through CHK2 activity (Sibon *et. al.,* 2000). Thus, if histones in slbp-RNAi embryos fail to be correctly incorporated into chromatin, it could lead to DNA damage and, through this, centrosome inactivation, as opposed to centrosome inactivation occurring as a direct effect of reduced SLBP levels. Centrosome inactivation is also related to nuclear fallout - nuclear fallout requires physical detachment of the nuclei from the cortex, and is likely to be promoted through loss of centrosomal-cortex interactions (Takada, Kelkar and Theurkauf, 2003; Sullivan, Fogarty and Theurkauf, 1993).

Thus, although the live phenotypes and the loss of accumulation of SLBP-interacting proteins to centrosomes exhibited by embryos in which SLBP function has been reduced could suggest additional functions for SLBP, the results presented so far are not formally inconsistent with the known role of SLBP in regulating histone protein levels.

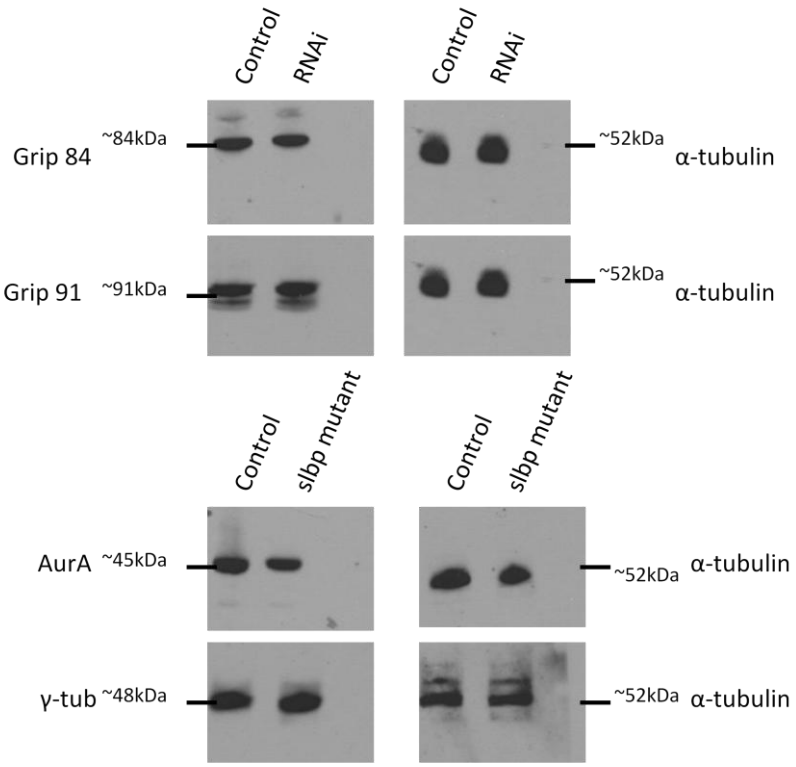


Figure 3-5. Levels of centrosomal interactor proteins remain the same in mutant or slbp-RNAi embryos. Western blots of control and either slbp mutant or slbp-RNAi 0-3h embryo extracts show levels of selected centrosomal SLBP-interactor proteins are not reduced in the absence of SLBP. (α-tubulin loading control)

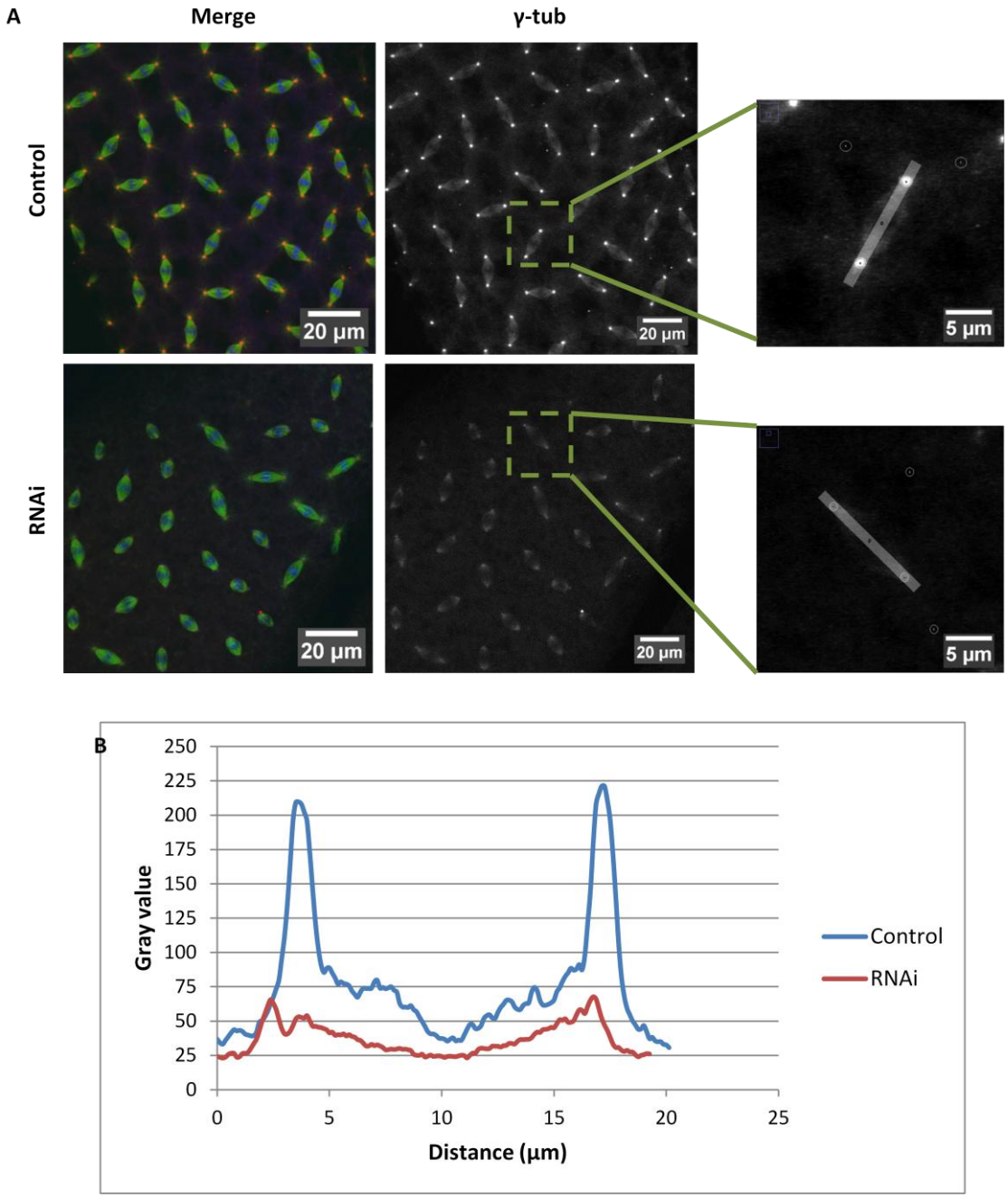


Figure 3-6.1. SLBP is involved in the centrosomal localisation of γ-tubulin. (A) 0-3h methanol fixed embryos stained for α-tubulin (Alexa 488), γ-tub (Alexa633) and DNA (Hoechst 33342). There is an observable decrease in signal intensity of γ-tubulin in slbp-RNAi embryos. (B) Line plots, taken through individual spindles, as displayed, highlight the mis-localistion away from the centrosomes (lower intensity at centrosomes in RNAi). (Highlighted dots in enlarged images represent sites that measurements for centrosome intensity were taken from)

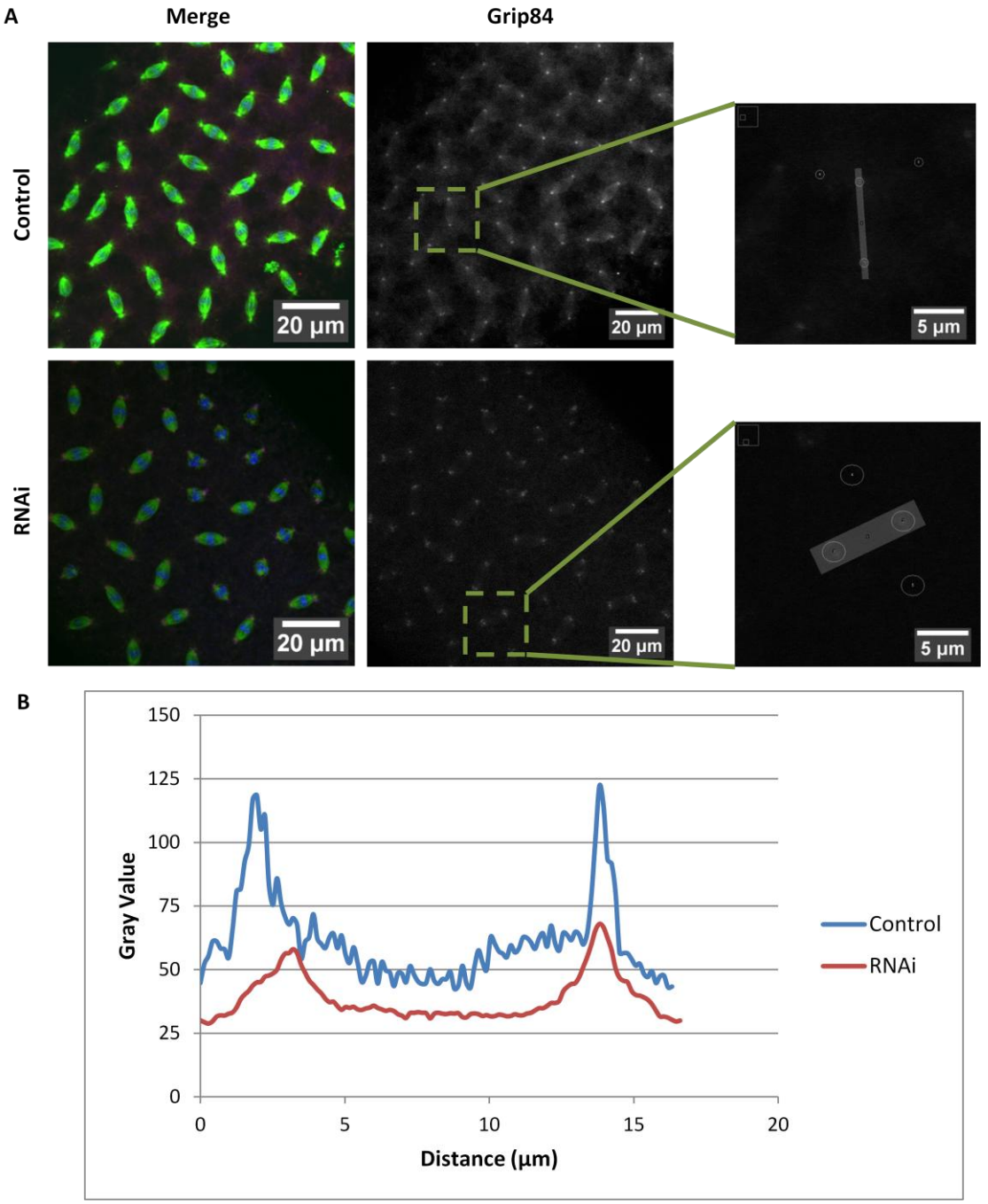


Figure 3-6.2. SLBP is involved in the centrosomal localisation of Grip84. (A) 0-3h methanol fixed embryos stained for α-tubulin (Alexa 488), Grip84 (Alexa633) and DNA (Hoechst 33342). There is an observable decrease in signal intensity of Grip84 in slbp-RNAi embryos. (B) Line plots, taken through individual spindles, as displayed, highlight the mis-localistion away from the centrosomes (lower intensity at centrosomes in RNAi). (Highlighted dots in enlarged images represent sites that measurements for centrosome intensity were taken from)

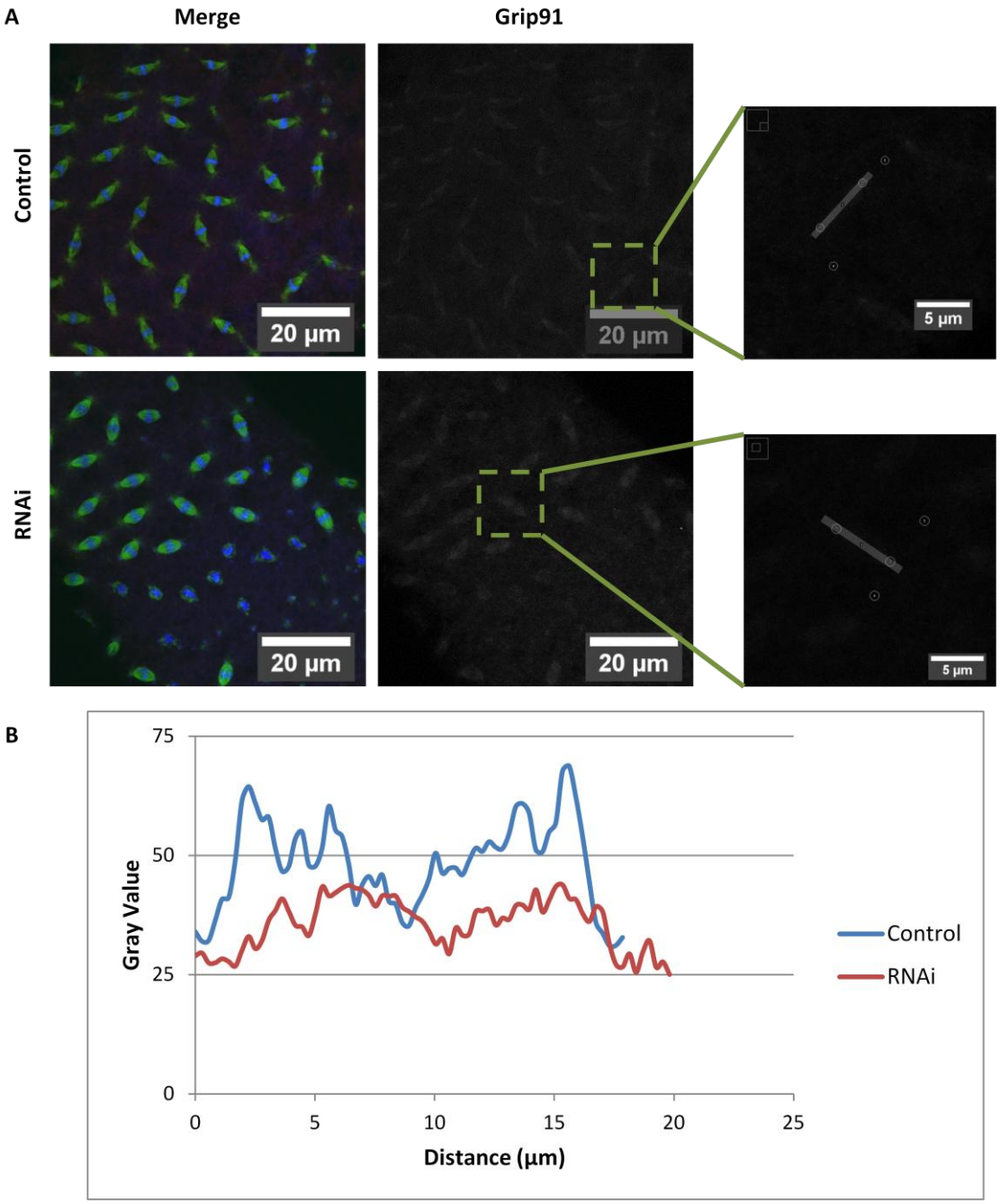


Figure 3-6.3 SLBP is involved in the centrosomal localisation of Grip91. (A) 0-3h methanol fixed embryos stained for α-tubulin (Alexa 488), Grip91 (Alexa633) and DNA (Hoechst 33342). Signal intensity of Grip91 is low in both conditions. (B) Line plots, taken through individual spindles, as displayed, show a slight decrease in intensity at centrosomes in the RNAi line, however this is not as clear as it was for Grip84 and γ-tubulin. (Highlighted dots in enlarged images represent sites that measurements for centrosome intensity were taken from)

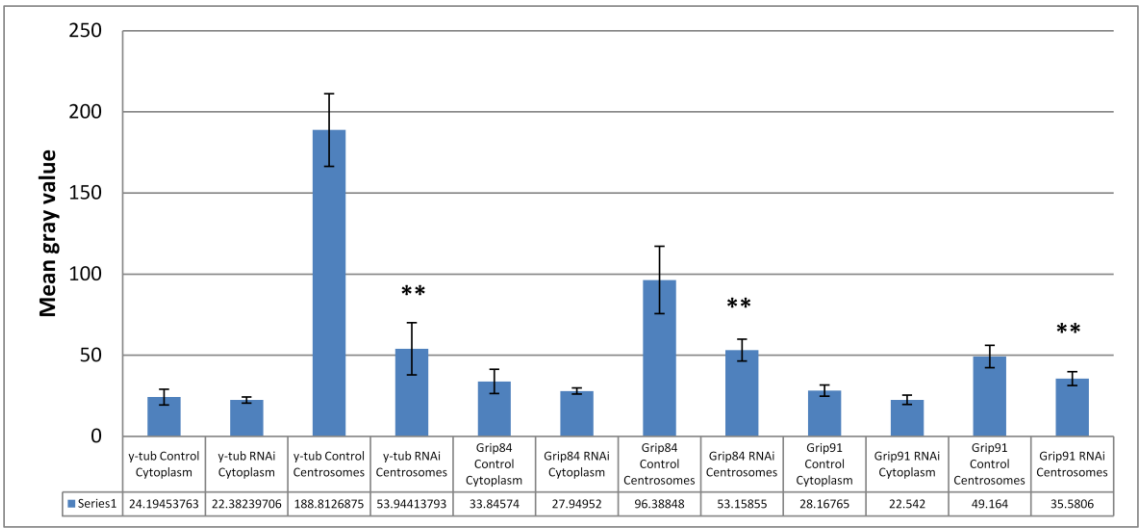


Figure 3-6.4 SLBP is involved in the centrosomal localisation of γ-TuSC proteins. Bar chart displaying signal intensities measured from the previous figures. Signal intensity of γ-TuSC proteins at the centrosomes was significantly reduced (T-test) in RNAi-slbp embryos compared to control. Background cytoplasmic signal was consistent.**p<0.01 n=39-93

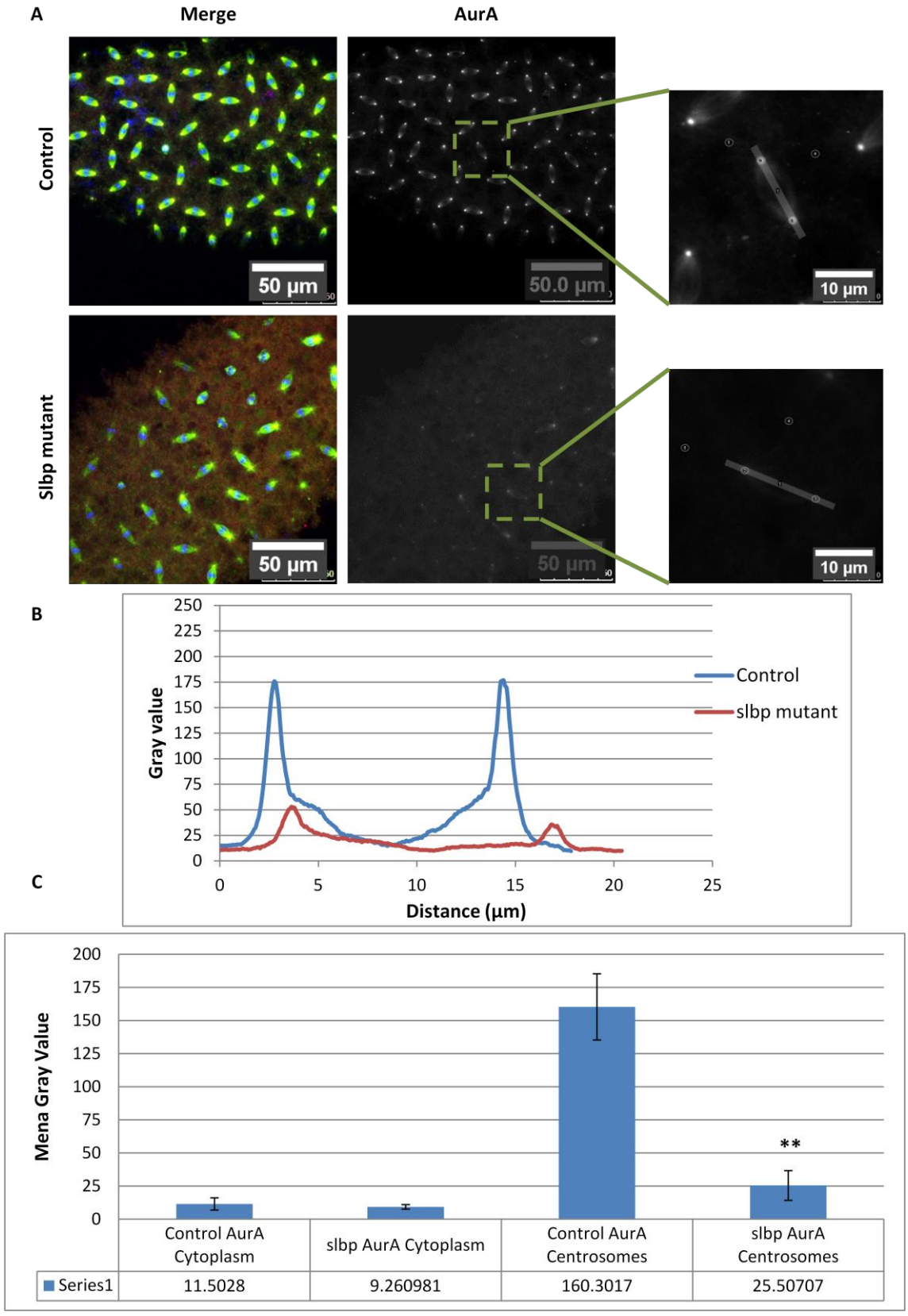


Figure 3-7. SLBP is also involved in the recruitment of AurA to the centrosomes. (A) 0-3h methanol fixed embryos stained for α-tubulin (Alexa 488), AurA (Alexa555) and DNA (Hoechst 33342). There is an observable decrease in signal intensity of AurA in the slbp mutant embryo. (B) A line plot, taken through individual spindles, as displayed, highlights the mislocalistion of

the protein away from the centrosomes (lower intensity at centrosomes). (Highlighted dots in enlarged images represent sites that measurements for centrosome intensity were taken from) (C) Bar chart displaying signal intensities measured from the above images. AurA signal at the centrosomes was significantly reduced (T-test) in slbp mutant embryos compared to control. Background cytoplasmic signal was consistent.**p<0.01 n=53-108

3.5. Reduction in SLBP causes increased DNA damage at interphase

To attempt to formally address whether all the phenotypes observed in slbp-RNAi embryos can be accounted for by a DNA damage response brought about by reduced histone levels, I went on to stain these embryos for the DNA damage marker γ-H2AV. Histone 2A variant (H2AV) is a variant histone protein that becomes phosphorylated upon double-stranded break (DSB) DNA damage, generating γ-H2AV (reviewed in Talbert and Henikoff 2010). One of the earliest responses (within 1 min) to DSB formation is the phosphorylation of S137 located in an SQ motif at the C-terminus of H2AV (Redon *et. al.,* 2002; Modesti and Kanaar, 2001; Rogakou *et. al.,* 1998); making it the gold-standard for DNA damage detection (Lake *et. al.,* 2013). Although a histone protein, H2AV is not regulated by SLBP as it does not form part of the poly-cistronic histone locus and its single gene copy within the *Drosophila* genome possesses a standard polyA tail (Mannironi *et. al.,* 1989).

First, I assessed the levels of γ-H2AV in 0-3 h old control and slbp-RNAi embryos via Western blotting. Surprisingly, this analysis showed lower levels of γ-H2AV in the RNAi line (Fig. 3-8, B) (cycling 0-3h embryos). A background level of DNA damage occurs naturally in early *Drosophila* embryos due to the rapid divisions that occur in the syncytium in the first 14 cycles. Many embryos imaged in the RNAi line presented with terminal arrest phenotypes (data not

shown), where structures have been degraded. Thus the total number of nuclei within a specific number of slbp-RNAi embryos is expected to be less than in the same number of control embryos; this may explain why a higher level of γ-H2AV is present via western blotting in control embryos. Interestingly, and in support of this, in addition to the major band at approx. 15kDa, relating to γ-H2AV, a number of other higher molecular weight proteins were identified using the antibody, presumably reflecting additional, cross-reacting epitopes (see below) or modified forms of γ-H2AV (for example, via ubiquitination). These higher bands also appear reduced in the slbp-RNAi line. As I have suggested that the lower abundance of γ-H2AV in slbp-RNAi embryos observed in my western blot is due to the fact that, when imaged, many embryos presented with a terminally arrested phenotype and so would possess fewer intact cellular structures/proteins, similarly the level of ubiquitination/phosphorylation would decrease in terminally arrested embryos. As a consequence of this, levels of modified forms of γ-H2AV would also appear reduced in the blot of slbp-RNAi embryos. To test this, and to prove that it is not a loading error, it would be interesting to probe the western blot with a phospho-specific antibody to observe if the level of all phosphorylated proteins decreased. If they did, this would support my theory that the reduced levels of γ-H2AV in slbp-RNAi embryos observed in my western blot are due to a large proportion of the embryos having terminally arrested.

To ensure the γ-H2AV antibody does indeed recognise γ-H2AV in DNA damaged nuclei, 0-3h old, methanol fixed control and slbp-RNAi embryos were stained for γ-H2AV and DNA and imaged using a confocal fluorescence microscope. Consistent with what is expected, I found that this antibody

specifically recognises a small proportion of nuclei within normal embryos (Fig. 3-8, A) - presumably reflecting those that have incorporated γ-H2AV due to DNA damage that occurs at low levels in control embryos. I also found that the γ-H2AV antibody additionally recognised centrosomes - presumably reflecting the additional epitopes identified by western blotting. As expected, a substantial proportion of slbp-RNAi embryos exhibited far greater numbers of γ-H2AV positive nuclei, supporting the notion that loss of SLBP leads to increased DNA damage in interphase (Fig. 3-8, A).

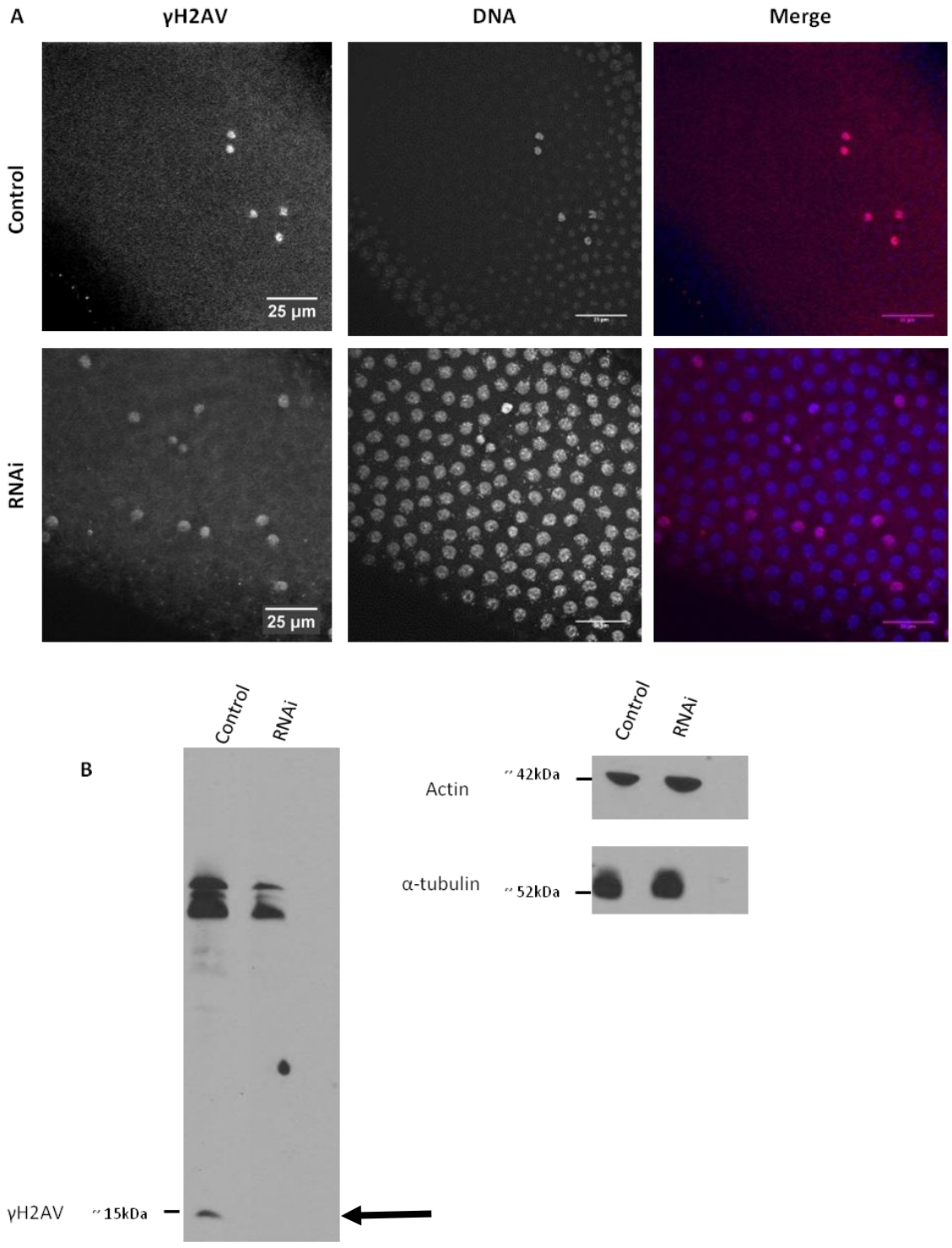


Figure 3-8. Reduction in SLBP causes increased DNA damage at interphase (A) 0-3h methanol-fixed control and slbp-RNAi embryos stained for γH2AV (Alexa633) and DNA (Hoechst 33342) and imaged at interphase. γH2AV antibody highlights areas of DNA damage. More nuclei are stained in the RNAi line. Nuclei in the control embryo are falling into the interior of the embryo, in the RNAi images the nuclei are still at the embryonic cortex. (B) Western blot analysis showing decreased levels of γH2AV protein in slbp-RNAi embryos (actin and α-tubulin loading controls).

3.6. slbp-RNAi mitotic phenotypes that occur in the absence of DNA damage suggest additional roles for SLBP in the early embryo

Although the proportion of nuclei possessing γ-H2AV staining was increased in some embryos in which SLBP function had been reduced through RNAi, I observed many mutant embryos possessing nuclei that had not incorporated γ-H2AV, but that presented mitotic phenotypes. A detailed analysis of these mitotic slbp-RNAi embryos, fixed and stained for γ-H2AV, EB1-GFP and DNA was therefore undertaken (Fig. 3-9 and Fig. 3-10). The additional centrosomal epitope/s recognised by the γ-H2AV antibody conveniently allowed centrosomes to be highlighted in these embryos.

I found that all the major slbp-RNAi phenotypes reported in Section 3.3 occur in the absence of DNA damage. For example, at metaphase, fragmented chromosomes with 'propellar-like' spindle formations surrounding them were seen; at telophase, elongated DNA barrels, where chromosomes have failed to divide properly, were observed, both in the absence of a γ-H2AV signal. Many slbp-RNAi embryos appeared to have doublet centrosomes at each pole. Due to time constraints I could not quantify the signal intensity of the DNA; however, since the intensity of DNA stain appears to be the same in RNAi and control lines, indicating similar quantities of DNA, this suggests the doubled centrosome number is not due to pseudo-endoreduplication (i.e. failed chromosome segregation in the previous cycle). Chromosome cohesion defects were also observed, with individual chromatids appearing to break away from aligned DNA. Finally, spindle orientation defects were also seen, which could be due to a problem in attachment to the cortex. Again, in all these cases, the phenotypes manifested in the absence of DNA damage, as assessed by γ-H2AV.

The above results are incompatible with a model in which the defects observed upon loss of SLBP are solely a consequence of histone loss-induced DNA damage and therefore are suggestive of additional roles for SLBP in the early embryo. Moreover, the phenotypes observed in slbp-RNAi embryos lacking DNA damage are consistent with the known functions of the SLBP-GFP interacting proteins. For example, the apparent centrosome duplication/cohesion defects observed can be explained by a failure to correctly regulate Asl or Sas-6, two core centriole biogenesis proteins (Azimzadeh and Marshall, 2010, Strnad *et. al.,* 2007), both of which were identified as SLBP interactors. Chromosome cohesion is maintained by the cohesin complex (reviewed in Haering and Jessberger, 2012; Oliveira and Nasmyth, 2010), at the core of which are two structural maintenance of chromosomes (SMC) proteins: SMC1 and SMC3 (Guacci, Koshland and Strunnikov, 1997; Michaelis, Ciosk and Nasmyth, 1997). Both of these were identified as SLBP-interactors in the MS analysis. Finally, spindle orientation and attachment to the cortical cytoskeleton are complex processes, but multiple proteins involved in this process were identified as interactors. Sponge (Spg) is important in formation of the pseudocleavage furrows that divide nuclei (Postner, Miller and Wieschaus, 1992); and Muskellin has a role in cell adhesion and cytoskeletal organisation in vertebrates (Adams, Seed and Lawler, 1998), the *Drosophila* ortholog is predicted to share a conserved mechanism of function (Adams, 2002).

In summary, the evidence obtained through analysis of the slbp-RNAi phenotype suggests an alternative model for SLBP function in the embryo, where SLBP is not limited to acting upon histone mRNA. In this alternative

hypothesis, SLBP has a separate role, binding a set of proteins with diverse, yet co-ordinated, roles in the embryo, related to the fast, synchronous mitotic divisions that occur - proteins with roles in centrosome duplication/organisation, chromosome cohesion and embryonic cortex function. Loss of SLBP would result in inactivation of these proteins and, through this, centrosome duplication defects, centrosome inactivation, mitotic spindle defects, spindle mis-orientation, loss of sister chromatid cohesion, abortive chromosome segregation and nuclear fallout.

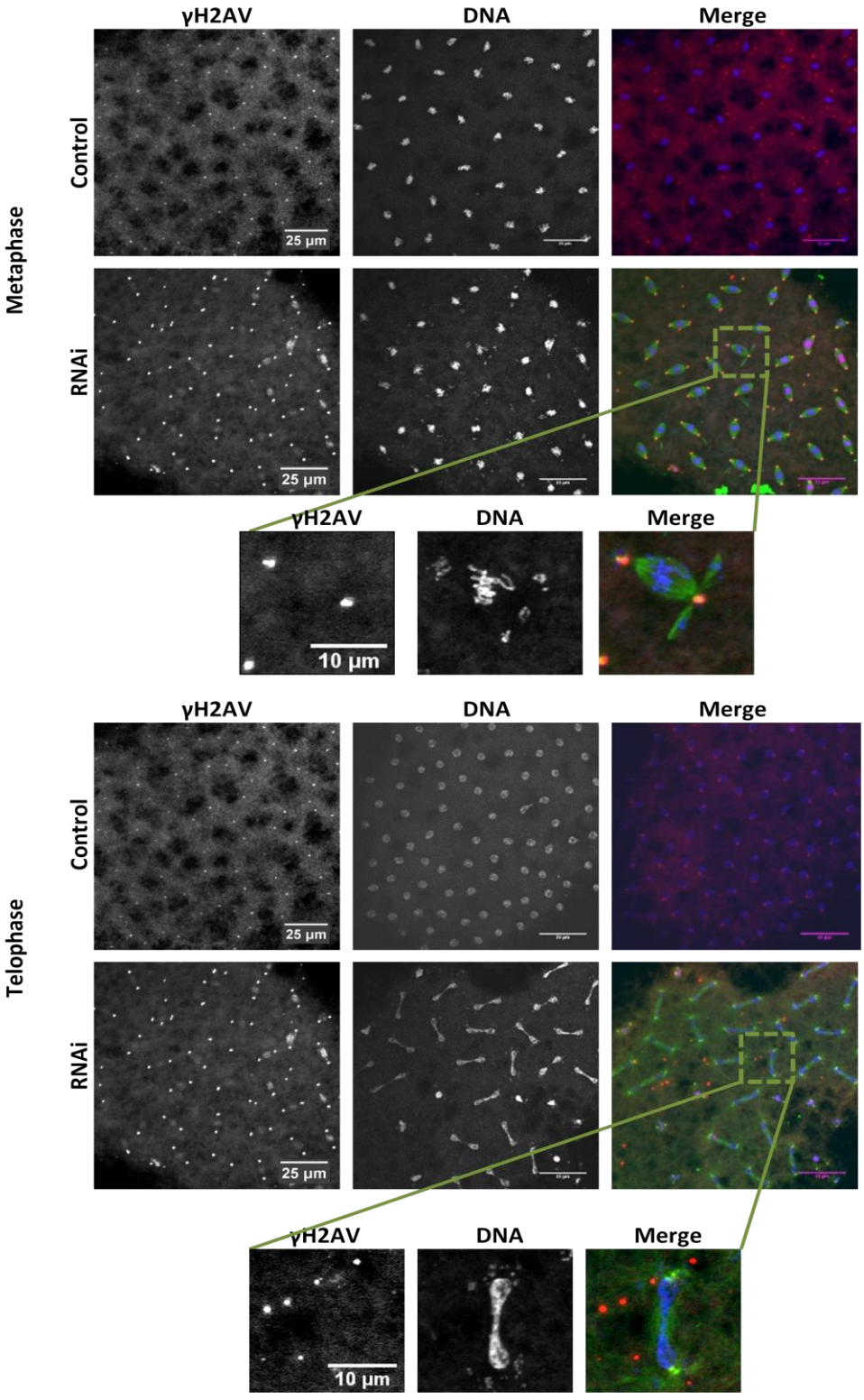


Figure 3-9. Reduced levels of SLBP cause chromosome defects that occur in mitosis in the absence of DNA damage. 0-3h methanol-fixed control and slbp-RNAi embryos stained for γH2AV (Alexa633) and DNA (Hoechst 33342) and imaged at mitotic stages (RNAi line maintains EB1-GFP through fixing and staining). Both at metaphase and telophase, clear chromosome defects can be seen occuring in the absence of γH2AV staining. γH2AV antibody also appears to stain centrosomes, this will be discussed later.

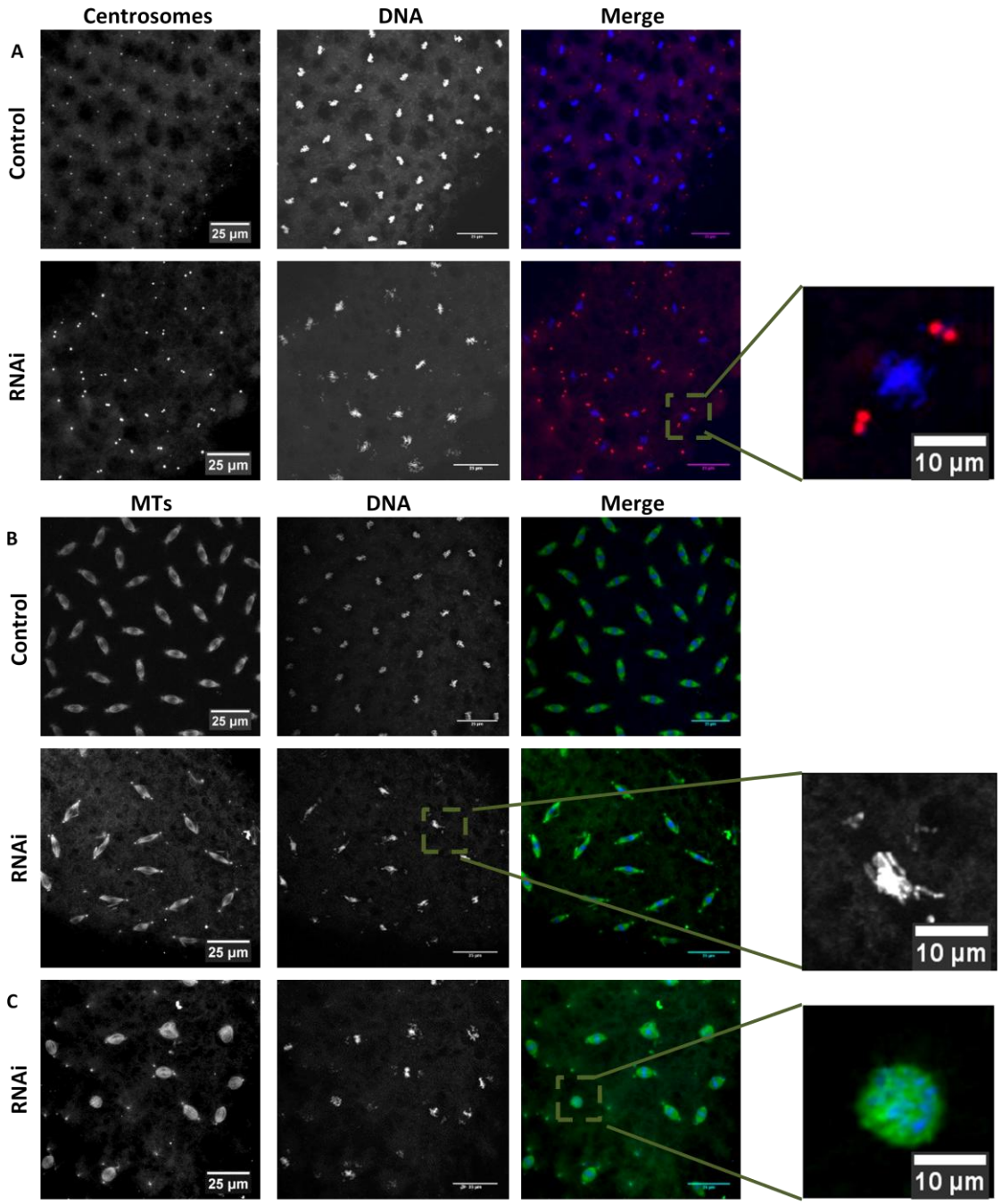


Figure 3-10. RNAi defects mirror phenotypes of SLBP interactors. 0-3h methanol-fixed control and slbp-RNAi embryos stained for γH2AV/centrosomes (Alexa633) and DNA (Hoechst 33342) (RNAi line maintains EB1-GFP through fixing and staining, as a control for this, embryos were stained for α-tubulin (Alexa488) and DNA (Hoechst 33342) (B)). (A) Doublet centrosomes can be seen in the RNAi line, representing a centrosome separation defect. DNA intensity appears the same indicating that doublet centrosomes are not present due to a previously failed division. (B) Individual chromatids can be seen broken off/separated from other aligned chromosomes which could be due to a failure in chromosome cohesion. (C) Some spindles

appear to be incorrectly orientated which could be caused by problems in attachment to the cortex.

3.7. Could SLBP act as a buffer to proteins targeted by CHK2?

Checkpoint kinase 2 (Chk2, or Mnk in *Drosophila*) is a key protein in the response to DNA damage within cells. Following DNA damage, CHK2 becomes phosphorylated and activated by ATM (ataxia telangiectasia-mutated), phosphorylating selected target proteins (Matsuoka *et. al.,* 2000). It has very recently been shown that *Drosophila* SLBP is a target of CHK2 in the early embryo (Iampietro *et. al.,* 2014). Phosphorylation of SLBP by CHK2 at residue S118, results in SLBP degradation. Moreover, another recent study by Takada, Collins and Kurahashi (2015) demonstrated that, in normal *Drosophila* embryos EGFP-tagged CHK2 localises to centrosomes, the nucleus, the inter-kinetochore region, the midbody and pseudocleavage furrows, but presumably remains inactive unless DNA damage occurs.

The correlation between CHK2 localisation and the reported localisations of our identified SLBP-interactors, in combination with the phenotype of slbp-RNAi embryos suggested to me a possible mode of action for SLBP in the early embryo. In this model (Fig. 3-11), high levels of SLBP, present throughout the embryo, act as a buffer to localised, basal CHK2 activity - CHK2 substrates at the centrosomes, chromatin and cortex cannot be phosphorylated by CHK2, as they are bound to SLBP. However, upon DNA damage, transient hyper-activation of CHK2 phosphorylates and degrades the surrounding SLBP, exposing phosphorylation sites on the substrates themselves. The inactivation of the substrates' functions leads to the classic DNA damage response - centrosome inactivation, mitotic spindle defects, spindle mis-orientation, loss of sister chromatid cohesion, abortive chromosome segregation and nuclear

fallout. In embryos in which SLBP levels are reduced, the buffering capacity of SLBP against CHK2 activity at these locations cannot be maintained - thus a DNA damage-like response occurs, though in the absence of DNA damage itself.

One intriguing piece of data that supports this hypothesis, relates to the centrosomal epitope staining of the γ-H2AV antibody. In 2013, it was found that in HeLa cells CHK2 functions downstream of DNA dependent protein kinase catalytic subunits (DNA PKcs) to phosphorylate H2AX (the human ortholog of H2AV) at Ser139 at its C terminus, during mitosis (Tu et al. 2013). The same site that ATM and ATR phosphorylate during DNA damage. The γ-H2AV antibody used in our experiments was generated against the CHK2 phosphorylated version of H2AV (Hawley, R.S. (DSHB Hybridoma Product UNC93-5.2.1)). Therefore, it is possible that this antibody recognises other phosphorylated CHK2 substrates, in addition to γ-H2AV. Careful comparison of control and slbp-RNAi embryos, fixed and stained with the γ-H2AV antibody, was undertaken. Imaging of fixed 0-3h embryos stained for γ-H2AV (Alexa633) and DNA (Hoechst 33342) showed an increase in signal intensity at centrosomes in the RNAi line, line plots taken through individual spindles highlight this (Fig. 3-12). Taking individual centrosomal and cytoplasmic measurements to perform statistical analysis revealed that this increase was significant (t-test $p<0.01$) (Fig. 3-12). This analysis demonstrates that the intensity of the centrosomal epitope is substantially and significantly increased in slbp-RNAi embryos - as would be expected if CHK2 were able to phosphorylate this epitope, in the absence of SLBP.

The above hypothesis relies on a subset of the SLBP interacting proteins being phosphorylated by CHK2. I therefore asked whether the SLBP interacting proteins possess consensus CHK2 phosphorylation sites. SLBP interactor proteins were scanned for the CHK2 consensus phosphorylation site described in Seo *et. al.* (2003), using ScanProsite (De Castro *et. al.,* 2006), along with a selected control group. Although an impressive 84% contained the sequence, 88% of control proteins also contained it (Fig. 3-13), suggesting that this sequence is not highly specific. Further investigation into this hypothesis needs to be made, as I will discuss later.

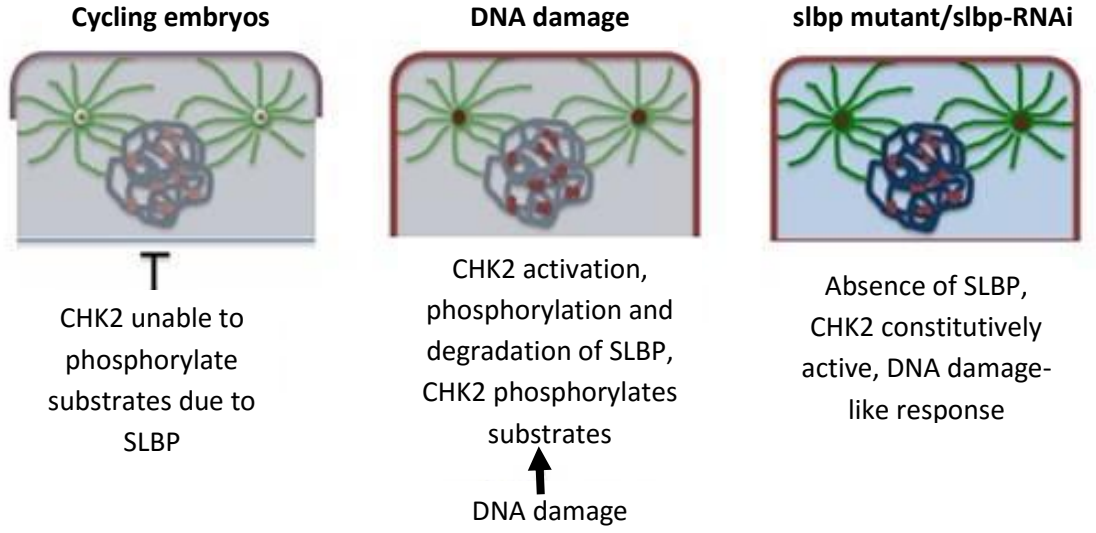


Figure 3-11. SLBP buffer hypothesis. In normal cycling embryos, SLBP binds interactor proteins, protecting them from CHK2 phosphorylation. Upon DNA damage, CHK2 phosphorylates and inactivates SLBP, allowing it to phosphorylate interactors and carry out the DNA damage response. In an slbp mutant/slbp-RNAi line CHK2 can access interactors uninhibited, resulting in a DNA damage-like response in the absence of DNA damage.

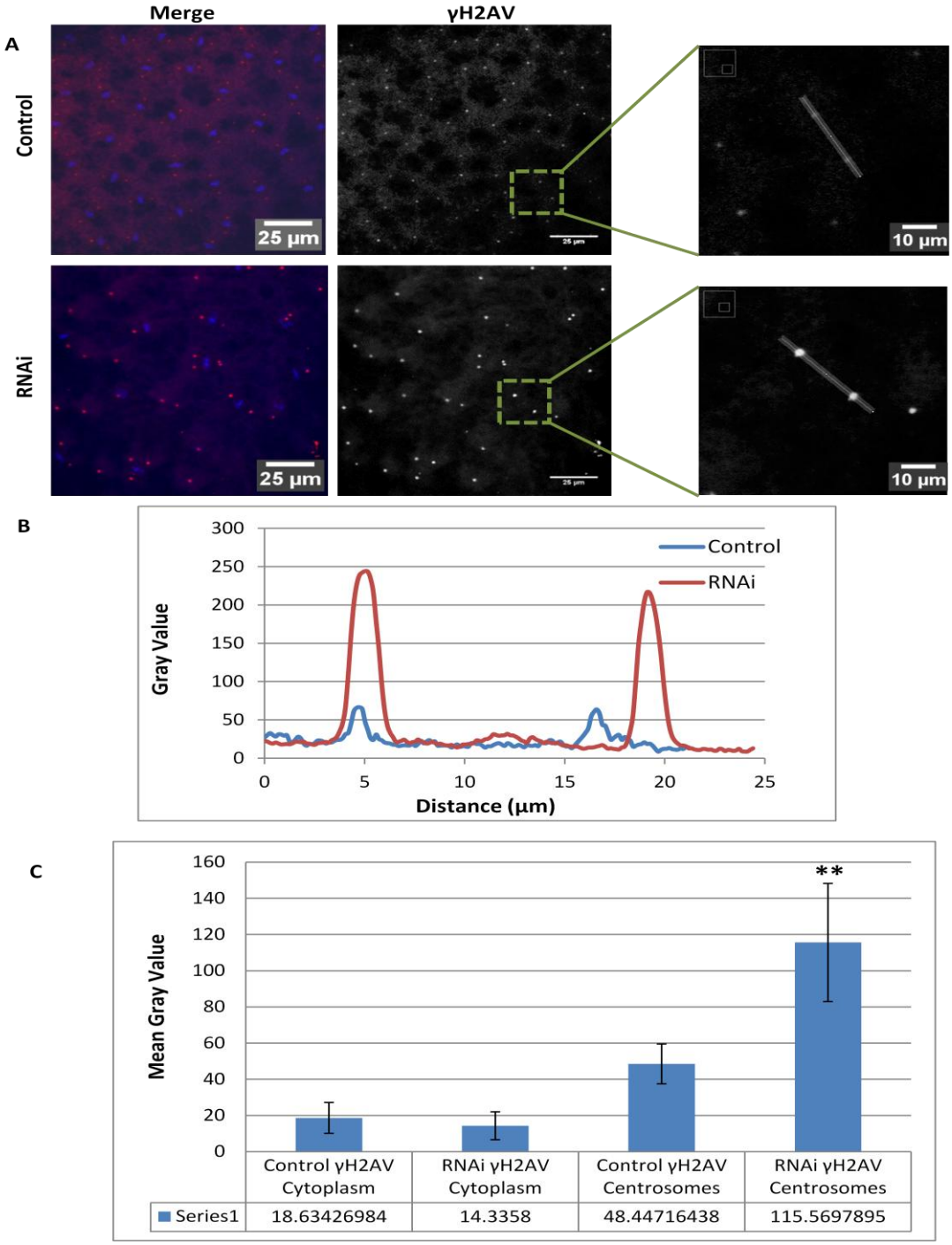


Figure 3-12. Reduced levels of SLBP causes an increased localisation of y-H2AV antibody to centrosomes. (A) 0-3h methanol-fixed control and slbp-RNAi embryos stained for γH2AV (Alexa633) and DNA (Hoechst 33342). Intensity of γH2AV signal is clearly increased in the RNAi line. (B) Line plots taken through single spindles, as displayed, show an increased localisation of the antibody to centrosomes in the RNAi line (higher intensity peaks). (C) Bar chart displaying signal intensities measured from the above images. γH2AV signal at the centrosomes was significantly increased (T-test) in slbp-RNAi embryos compared to control. Background cytoplasmic signal was consistent. **p<0.01 n= 35-73

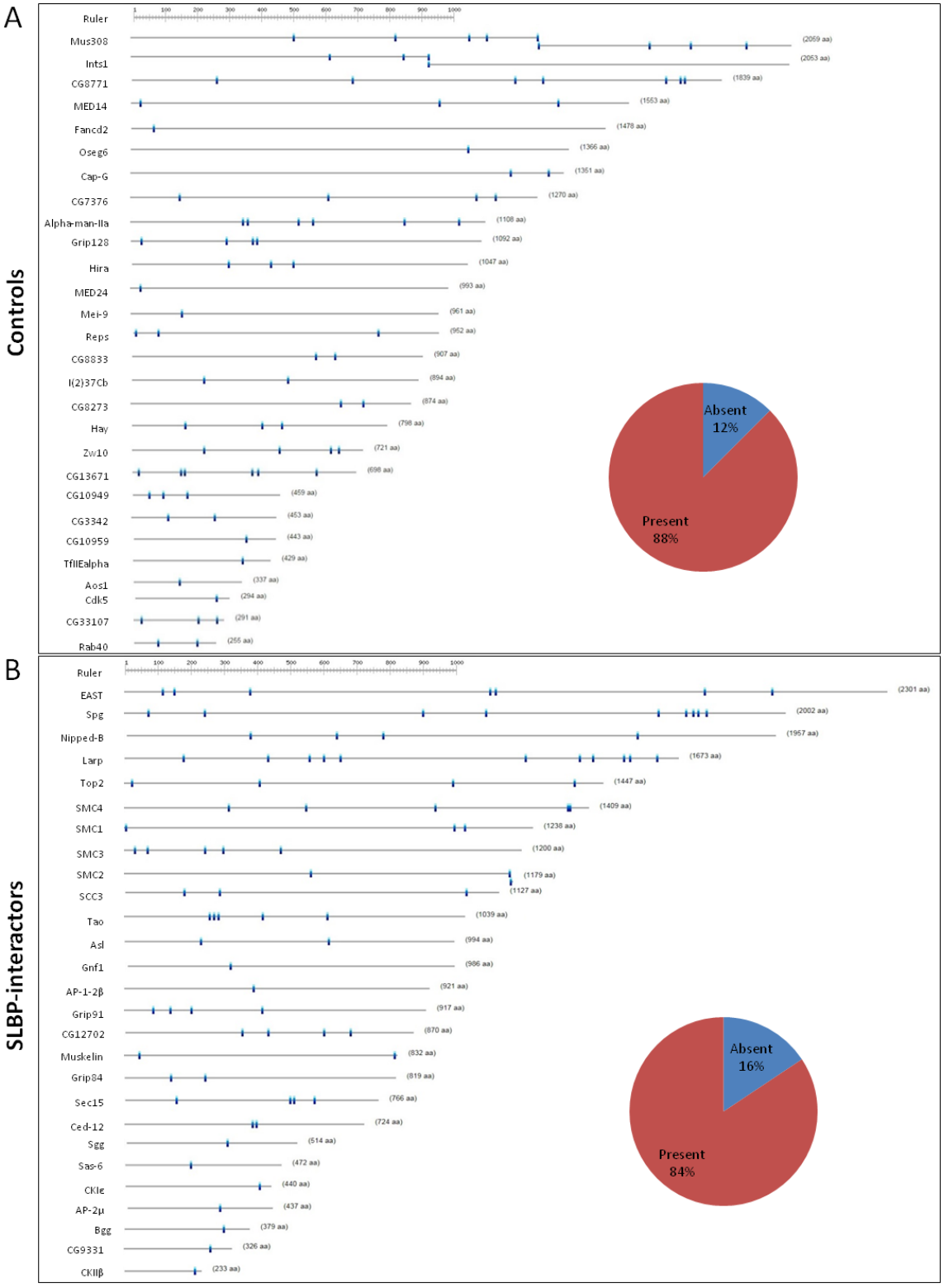


Figure 3-13. There is no enrichment of CHK2 consensus phosphorylation sites in SLBP-interactors. SLBP-interactor proteins, and a randomly selected control group with a similar molecular weight distribution, were screened for the CHK2 consensus site described in Seo *et. al.* (2003). 88% of control proteins, compared to 84% of SLBP-interactor proteins, contained at least 1 site.

3.8. Conclusion

In summary, the data described here presents a case for additional functions of SLBP. I have shown that SLBP associates with a large number of proteins, enriched for specific co-ordinated cellular functions, independently of associating with their mRNAs. Loss of SLBP causes mis-localisation of at least some of the centrosomal interactor proteins, as well as multiple mitotic defects. These defects cannot directly be explained by a lack of histone protein, and are not due to DNA damage - although γ-H2AV staining does reveal higher levels of damage in interphase nuclei lacking SLBP, during mitosis defects occur in the absence of DNA damage; defects that correspond to the known functions of the SLBP-interacting proteins identified. I provide a hypothesis to explain how SLBP could function in relation to so many different proteins with different functions, proposing that it acts as a cellular protein buffer, protecting potential CHK2 kinase substrates from phosphorylation by basally-activated CHK2. Although there is, as yet, no substantial evidence to support this hypothesis, I will go on to discuss a series of experiments that will, hopefully, test it.

4. Discussion

In this Masters by research project I aimed to a) investigate additional roles that SLBP may play outside of it's know function in processing histone mRNAs, and b) determine whether SLBP acts a mitotic MAP, following inconclusive results during my undergraduate project.

Results showed that SLBP does not bind MTs during mitosis, and therefore I conclude that it does not function as a mitotic MAP. RNAi experiments demonstrated that loss of SLBP causes multiple mitotic defects, which mirror the DNA damage response. However, staining with γ-H2AV showed that, in many of instances, these defects occur in the absence of DNA damage. Defects mirrored the phenotypes of proteins identified as SLBP interactors during my undergraduate work and in slbp-RNAi embryos, mis-localisation of some of these proteins was observed. Therefore, I conclude that SLBP has additional functions to histone pre-mRNA processing, which involve interactions with the identified proteins. I also suggest a model for how SLBP may perform these functions; acting as a buffer against CHK2 phosphorylation of interactors. This hypothesis and future experiments to test it are discussed further below.

4.1. SLBP is not a mitotic MAP

Previous work carried out during my undergraduate project failed to conclude whether SLBP was, indeed, a mitotic MAP as was originally proposed, as although a weak localisation of SLBP-GFP was seen around the spindle area in live imaging, biochemical association was not proven (Supplementary materials). A microtubule co-sedimentation assay, using SLBP-GFP expressing embryos, did show some GFP in the +taxol pellet, indicating association with

the MTs that sedimented there, however GFP was also present in the +taxol supernatant, making results un-reliable.

Therefore in this project I repeated the microtubule co-sedimentation assay using MG132-treated SLBP-GFP expressing embryos. In the previous experiment cycling embryos were used and so many of them would not have been in mitosis. MG132 arrests embryos before the metaphase-anaphase transition by inhibiting the 26S proteosome (Genschik *et. al.,* 1998). The result in this thesis showed clearly that SLBP-GFP does not pellet with MTs in the +taxol pellet (Fig. 3-1) and I concluded that SLBP is not a mitotic MAP.

The initial reason for investigating SLBP as a mitotic MAP was due to previous work by the Wakefield laboratory (unpublished). Triplicate MT co-sedimentation assays, followed by mass spectrometry analysis, comparing cycling and mitotic embryos, identified a number of proteins that were enriched on MTs during mitosis. One of these was SLBP. Why, then, does SLBP-GFP not co-sediment with MTs in the assay? There are two main possibilities; first, it is possible that the GFP-tag partially interferes with the biochemical properties of SLBP and that, in vivo, SLBP does bind to MTs. Alternatively, it is possible that SLBP was identified as a "false positive" in the original MS. In support of the latter, since starting this project the MS data has been re-analysed and SLBP does not get selected with the more stringent, criteria currently used in the laboratory. (Stacey Scott MbyRes thesis, 2015).

4.2. SLBP only regulates histone mRNAs

SLBP's known function of processing histone pre-mRNAs has been well characterised (Sullivan *et. al.,* 2001) and, in fact, histone mRNAs have been found to be the only mRNAs that SLBP associates with in human tissue culture (Townley-Tilson *et. al.,* 2006). Therefore, the finding in my undergraduate project, via mass spectrometry analysis, that SLBP associates with a large number of proteins was very interesting. Although MS identifies proteins, and not mRNAs, it was important to investigate further if SLBP associates with the interactor protein mRNAs; one hypothesis was that the proteins were identified by MS indirectly due to SLBP binding their nascent mRNAs in the process of translation. This hypothesis was supported by the observation that the second highest scoring interactor was La motif-related protein (Larp), a protein with a known function in regulating mRNAs containing a 5'TOP (Aoki *et. al.,*2013). Therefore, it was deemed possible that SLBP and Larp might work together to regulate translation of a subset of mRNAs, corresponding to the SLBP-interacting proteins identified via MS.

To test this, I identified the mRNAs associated with GFP-SLBP IPs and quantified their abundance. This RNA-Seq analysis showed that the only mRNAs enriched in an SLBP-GFP IP sample were histone mRNAs (Fig. 3-2). However, it is worth noting that, due to time constraints, I was unable to perform a technical control or duplicates. As a technical control I would have liked to perform a parallel IP using just GFP-TRAP-A beads to identify enrichment of non-specific binding RNAs. In addition, as I have proposed that SLBP could interact with mRNAs in the process of translation, it is worth considering that is is possible that such mRNAs may not have come down in the IP due to their weight, and so would not appear in the RNA-Seq. Nonetheless, western blot

analysis of the protein levels of some of the interactors in an RNAi-slbp/mutant line revealed no change from control levels (Fig. 3-5). If SLBP did possess a function with these mRNAs, the levels of translation would be affected in an RNAi line and consequently, so would protein levels. This data supports previous work that has shown that SLBP only interacts with histone mRNAs. Therefore I must conclude that SLBP associates with identified interactors at a protein:protein level.

4.3. SLBP has previously uncharacterised mitotic functions

Live and fixed imaging of embryos derived from flies expressing a shRNA specific for the Slbp gene in the female germline, showed multiple mitotic defects. I observed centrosome inactivation, acentrosomal spindle assembly, nuclear fallout, spindle orientation defects and chromosome cohesion failures in these embryos, consistent with the previously published fixed analysis of slbp mutant embryos (Sullivan *et. al.,* 2009). The question I have attempted to answer in this thesis is whether all these defects can be attributed to SLBP's role in histone mRNA processing, or whether it is possible that SLBP performs other roles in the *Drosophila* early embryo.

Work by Sullivan *et. al.* (2009) has demonstrated that in *Drosophila* U2OS cells, knockdown of SLBP by RNAi causes reduced levels of all 4 core histones. This is consistent with work in this thesis that shows that slbp-RNAi embryos possess reduced levels of H2B and H3, via western blot analysis (Fig. 3-5), and a study by Iampietro *et. al.* (2014) that identified reduced levels of H3 and H4 in slbp mutant *Drosophila* embryos. The work by Sullivan *et. al.* (2009) also characterised the slbp-RNAi cells to have a decreased rate of DNA synthesis, accumulation of cells in S-phase and retention of histone mRNAs in the nucleus

(defining its role in export of histone mRNAs from the nucleus). Further to this, Salzler *et. al.* (2009) used slbp null mutant embryos to show that loss of SLBP results in genomic instability: increased chromosome breaks, loss of heterozygosity, tetraploidy and position effect variegation. They attribute this to the rate of histone protein expression and its timing in relation to the cell cycle being perturbed. The accumulated results of these two papers could go some of the way in explaining the phenotype we see. Reduced levels of histone could cause DNA damage, invoking the DNA damage response and the mitotic effects we observed.

However, a paper by Günesdogan, Jäckle and Herzig (2014) showed that *Drosophila* histone null mutant embryos, which lacked all canonical histones, had a prolonged S-phase and cells arrested in G_2 in the absence of DNA damage. This suggests that loss of histones does not contribute to DNA damage and therefore the effects on embryonic mitosis that I report here are not a consequence of a DNA damage response. However, it must be acknowledged that this conclusion rests on only one publication, and that it is probable that loss of histones contributes to DNA damage, if indirectly through de-stabilising chromosomes. The strongest piece of evidence that the mitotic phenotypes observed in slbp-RNAi embryos are not a consequence of histone-induced DNA damage comes from staining embryos for the DNA damage marker γ-H2AV. Fixed slbp-RNAi embryos do accumulate more γ-H2AV-positive interphase nuclei than their wild type counterparts; however, many mitotic embryos had clear chromosome, centrosome and spindle defects that occurred in the absence of DNA damage, as attributed by a lack of γ-H2AV signal (Fig. 3-8, Fig. 3-9 and Fig. 3-10). Therefore, even though interphase

levels of DNA damage are higher in RNAi embryos, mitotic defects can happen in the absence of damage and therefore likely represent a DNA damage-independent response. Together this data suggests that there must be an alternative cause for the mitotic defects observed in embryos lacking SLBP.

γ-H2AV is the ideal antibody for studying DNA damage. It has been used in previous experiments in *Drosophila*, including in relation to changes in levels of the canonical histones (Günesdogan, Jäckle and Herzig, 2014) so is not affected by altered histone levels. It is not regulated by SLBP, as other histone proteins are, as it possesses a polyA-tail (Mannironi *et. al.,* 1989), and it has been described as the 'gold standard' of DNA damage detection (Lake *et. al.,* 2013).

Although the defects observed in the slbp-RNAi embryos that I have reported here can occur in the absence of DNA damage, the phenotype is reminiscent with a DNA damage-like response. Sibon *et. al.* (2000) carried out an investigation into DNA-damage dependent centrosome inactivation in *Drosophila* embryos*.* They discovered that checkpoint mutant embryos (grp and mei-41 (or chk1 and rad3)) accumulate chromosome segregation defects caused by centrosome inactivation, and that this centrosome inactivation involves the failure of γ-TuSC components γ-tubulin, Grip84 and Grip91 to be recruited to centrosomes. This phenotype is also observed upon reduction of SLBP; slbp-RNAi embryos show centrosome inactivation through live imaging (Fig. 3-4), and mis-localisation of γ-TuSC components and AurA through fixed imaging and analysis (Fig. 3-6.1, Fig. 3-6.2, Fig. 3-6.3, Fig. 3-6.4 and Fig. 3-7).

Centrosome inactivation can result in chromosome segregation defects and subsequent selection of defective nuclei for nuclear fallout, another phenotype presented in our RNAi line (Fig.3-4). Nuclear fallout has been described as a response to grp mutation in *Drosophila* (Sullivan, Fogarty and Theurkauf, 1993), as well as being a consequence of centrosome inactivation that results in chromosome mis-segregation (Takada, Kelkar and Theurkauf, 2003).

Finally, the acentrosomal spindle assembly we observed is also likely to be, at least in part, a consequence of centrosome inactivation (Fig. 3-4). There are multiple ways to build a spindle (Duncan and Wakefield, 2011), so even when centrosomes become inactivated, for example in response to DNA damage, spindles can form through these other mechanisms.

The data presented here therefore suggests that embryos in which SLBP levels have been reduced, via RNAi, elicit a phenotype similar to that seen upon DNA damage, even in nuclei that show no DNA damage. How, then, might lack of SLBP cause such a phenotype?

I propose that the answer relates to the SLBP-interacting proteins I identified in my undergraduate project. Although slbp-RNAi embryos phenocopy a DNA damage response phenotype, they also phenocopy embryos lacking SLBP interactor proteins. One group of interactors have functions at the centrosomes: Grip84, Grip91, AurA, Asl, and Sas-6. Grip84 and Grip91, as I have already explained, are part of the γ-TuSC. Asl and Sas-6 are core centriolar components (Varmark *et. al.*, 2007; Leidel *et. al.,* 2005; Dammermann *et. al.,* 2004). AurA is a kinase that works in combination with centrosomin (CNN) to

recruit γ-tubulin to centrosomes (Terada, Uetake and Kuriyama, 2003). Loss of function of these proteins could result in centrosome inactivation as we saw in our slbp-RNAi line (Fig. 3-4), and as supported by Sibon *et. al.* (2000).

In addition to centrosome inactivation, centrosome duplication defects were observed in fixed staining with a γ-H2AV antibody (Fig. 3-10). Western blot analysis of γ-H2AV levels in control vs. RNAi embryos revealed higher molecular weight bands that the antibody recognised (Fig. 3-8), and staining with the antibody revealed that it conveniently identified centrosomes, in addition to sites of DNA damage. This allowed me to visualise centrosome duplication problems that occurred in RNAi embryos (Fig. 3-10). Doublet centrosomes were apparent, which could be caused either by unregulated centrosome duplication or a failed previous anaphase. I believe that it is not the latter, as in this case double the amount of DNA would be present and Hoechst 33342 signal for DNA appeared similar in both control and RNAi, although this was not quantified due to time constraints. Therefore, it is likely that this phenotype represents uncontrolled centrosome duplication. Supernumerary centrosomes have previously been reported to be a result of p53 mutation in mouse embryonic fibroblasts (Fukasawa *et. al.,* 1996), suggesting a role for p53 in control of the process; but also as a consequence of centrosome inactivation (Löffler *et. al.,* 2006).

The second group of SLBP interactors have roles in chromosome cohesion: SMC1, SMC2, SMC3, SMC4, NippedB/SCC2, SCC3 and Top2. SMC1, SMC3 and SCC3 make up the core components of the cohesin complex in *Drosophila* (Rollins *et. al.,* 2004), which plays a crucial role in binding sister chromatids

together before anaphase. NippedB/SCC2 has been found to mediate binding of the cohesin complex to chromatin (Ishiguro and Watanabe, 2007). SMC4 and SMC2 are components of the condensin complex, important for chromosome condensation, and Top2 is an enzyme that facilitates the condensin complex (Charbin, Bouchoux and Uhlmann, 2014). Problems in chromosome cohesion were observed in our slbp-RNAi line, made clear through fixed staining (Fig. 3-10). Individual chromatids could be seen 'breaking away' from the aligned mass at the equator during metaphase. This could be as a consequence of failing to correctly localise the chromosome cohesion proteins identified as interactors, similar to the mis-localisation of the centrosomal protein interactors Grip84, Grip91 and AurA in our RNAi line (Fig. 3-6.2, Fig. 3-6.3 and Fig. 3-7). Unfortunately due to time restrictions, localisation studies of chromosome cohesion proteins have not yet been carried out.

The final ontology grouping of SLBP interactors have functions at the cortex: Spg, ELMO, Sgg, CKIε, AP-2μ, AP1-2-β, Muskelin, Sec15 and CG6617. Slbp-RNAi embryos show spindle orientation defects (Fig. 3-10), which could be as a result of failure to attach to the cortex. Spindle:cortex attachment is a complex multi-step process. Spg is known to be important in formation of pseudo-cleavage furrows that separate individual spindles and spg mutants show catastrophic nuclear fallout (Postner, Miller and Wieschaus, 1992). Muskellin has a role in cell adhesion and cytoskeletal organisation in vertebrates (Adams, Seed and Lawler, 1998), and the *Drosophila* ortholog is predicted to share a conserved mechanism of function (Adams, 2002). Therefore it is possible that the spindle orientation defects we see are a consequence of problems with these identified cortex proteins.

The data in this thesis is therefore consistent with the hypothesis that the mitotic phenotype observed upon RNAi-mediated loss of SLBP is a result of mis-localisation/inactivation of the identified interactor proteins, and not as a result of the DNA damage response. The question then is how SLBP regulates such a large number of proteins with such diverse functions.

I hypothesise that it occurs via a shared mechanism of function with the DNA damage response kinase CHK2. Previous research has shown that in *Drosophila* embryos CHK2 phosphorylates SLBP, at residue S118, in response to DNA damage, causing degradation of SLBP, nuclear retention of histone mRNAs and nuclear fallout (Iampietro *et. al.,* 2014). Further to this, CHK2-GFP (Mnk in *Drosophila)* has been found to localise to centrosomes, the mid-body, pseudo-cleavage furrows, nuclei and inter-kinetochore regions; all locations that mirror our identified interactors (Takada, Collins and Kurahashi, 2015). Presumably CHK2 is held inactive in these locations until DNA damage is detected, to allow normal cell function; otherwise a DNA damage-like response would occur in all embryos.

I therefore hypothesise that SLBP acts as a buffer to interactor proteins against low, basal CHK2 activity in these locations during normal embryo development. In nuclei / embryos in which DNA damage occurs, however, CHK2 activity would be transiently increased, phosphorylating SLBP, degrading it and allowing CHK2 access to the interactor proteins, eliciting the DNA damage response. If this were the case, then in our slbp-RNAi embryos, basally active CHK2 would be able to access interactor proteins uninhibited by SLBP, resulting in the DNA damage-like phenotype observed (Illustration in Fig. 3-11).

It should be noted that SLBP does not need to interact directly with all the proteins identified as interactors - many of them are already characterised as functioning in complex with one another (e.g. Grip84 and Grip91; ELMO and Spg; the Cohesin complex). It is therefore possible that SLBP only buffers specific proteins within the complexes identified, and that the others were identified in the IP as they were pulled down with this interacting subunit.

4.4. Investigating our SLBP buffer hypothesis

One other piece of evidence in this thesis supports the SLBP buffer hypothesis - during my studies I noticed that the anti-γ-H2AV antibody recognised centrosomes; and that in the slbp-RNAi embryos, this centrosomal staining was dramatically increased (t-test $p<0.01$) (Fig. 3-12). Western blot analysis of extracts, using this γ-H2AV antibody, demonstrated the presence of higher molecular weight epitopes, in addition to the band corresponding to γ-H2AV at 15kDa (Fig. 3-8 and Fig. 3-12). The antibody was made against the phosphorylated version of H2AV (deposited to the DSHB by Hawley, R.S.). It therefore seems likely that this antibody recognises not only γ-H2AV, but also other phosphorylated CHK2 substrates. If this is the case, then our data shows that CHK2 substrates at the centrosome become increasingly phosphorylated in the absence of SLBP.

Finally, to attempt to test whether CHK2 phosphorylates some of our SLBP interacting proteins, I screened the interactors for the presence of the CHK2 consensus sequence (Seo *et. al.,* 2003), using the online database ScanProsite (De Castro *et. al.,* 2006); however, results showed that there is no increase in CHK2 consensus phosphorylation sites in our SLBP interactors, compared to

the control group (Fig. 3-13). This may be due to the fact that not all identified interactors need to bind SLBP directly, and therefore not all the interactors need to be CHK2 substrates. Therefore, currently although my work is consistent with our buffer hypothesis we require additional evidence.

4.5. Future work

As an initial step, reciprocal IPs with antibodies for SLBP-interacting proteins should be performed to confirm their association with SLBP. This could be carried out either using extracts from wild type embryos or embryos expressing SLBP-GFP, western blotting with anti-SLBP or anti-GFP, respectively. Association of SLBP could also be verified via MS.

Secondly, it would be good to repeat the localisation experiments that I carried out for centrosomal proteins, on interactor proteins of other ontologies, to support evidence that loss of SLBP affects the localisation of interactor proteins. This would also support that the chromosomal and spindle orientation defects we observed in our RNAi line are a consequence of mis-localisation/inactivation of SLBP interactors.

Thirdly, and crucially, it would be beneficial to create a new antibody (Ab) against SLBP. The antibody used in this project was obtained from Iampietro *et. al.* (2014) and is a serum, not a purified antibody. In addition to SLBP it recognises additional epitopes (Fig. 3-3) and so cannot be used for immuno-staining or antibody injection experiments, due to its lack of specificity. Immuno-staining with an SLBP Ab would mean we could see greater detail of SLBP's cellular localisation during mitosis, compared to the live SLBP-GFP imaging I

have already carried out (Supplementary materials). This would allow us to see if SLBP localises to centrosomes, the mid-body, pseudo-cleavage furrows, nuclei and inter-kinetochore regions, as both CHK2 and SLBP-interactors do.

With an SLBP Ab it would also be possible to carry out Ab injections into wild-type embryos. This would allow acute inhibition of SLBP, disrupting SLBP function at different stages of the cell cycle. It would therefore allow us to unequivocally test whether the mitotic phenotypes observed are independent of S phase (i.e. DNA replication and histone loading) - any phenotype observed after injection of antibodies as nuclei enter mitosis, at nuclear envelope breakdown, could not be due to histone defects.

Finally, more work is needed to explore our SLBP buffer hypothesis. One way to do this would be to verify the cross-specificity of the γ-H2AV Ab. An IP using the γ-H2AV Ab would precipitate anything that the antibody recognises. This sample could then be subjected to MS and the cross-reacting proteins identified. Based on our hypothesis, we would expect at least some of these epitopes, and therefore some of the γ-H2AV interactors, to be the SLBP interacting proteins originally identified.

In addition, genetic experiments could be used to confirm the relationship between SLBP and CHK2. This could be done in two ways. Firstly, we could cross a constitutively active CHK2 (CA-CHK2) mutant with a line which over-expresses SLBP. We would expect a DNA damage-like response in a CA-CHK2 mutant; however, if our buffer hypothesis is correct, over-expressing SLBP should quench CHK2 activity and rescue the phenotype. Conversely, as

we have observed the phenotype of our slbp-RNAi line, we could cross this with a chk2 mutant line to observe whether this rescues the phenotype. We would expect, if our buffer hypothesis is correct, that the effects we see in our slbp-RNAi line are due to CHK2 activity. Therefore, we would expect that this phenotype would be rescued or partially rescued when CHK2 protein levels are reduced in the same line.

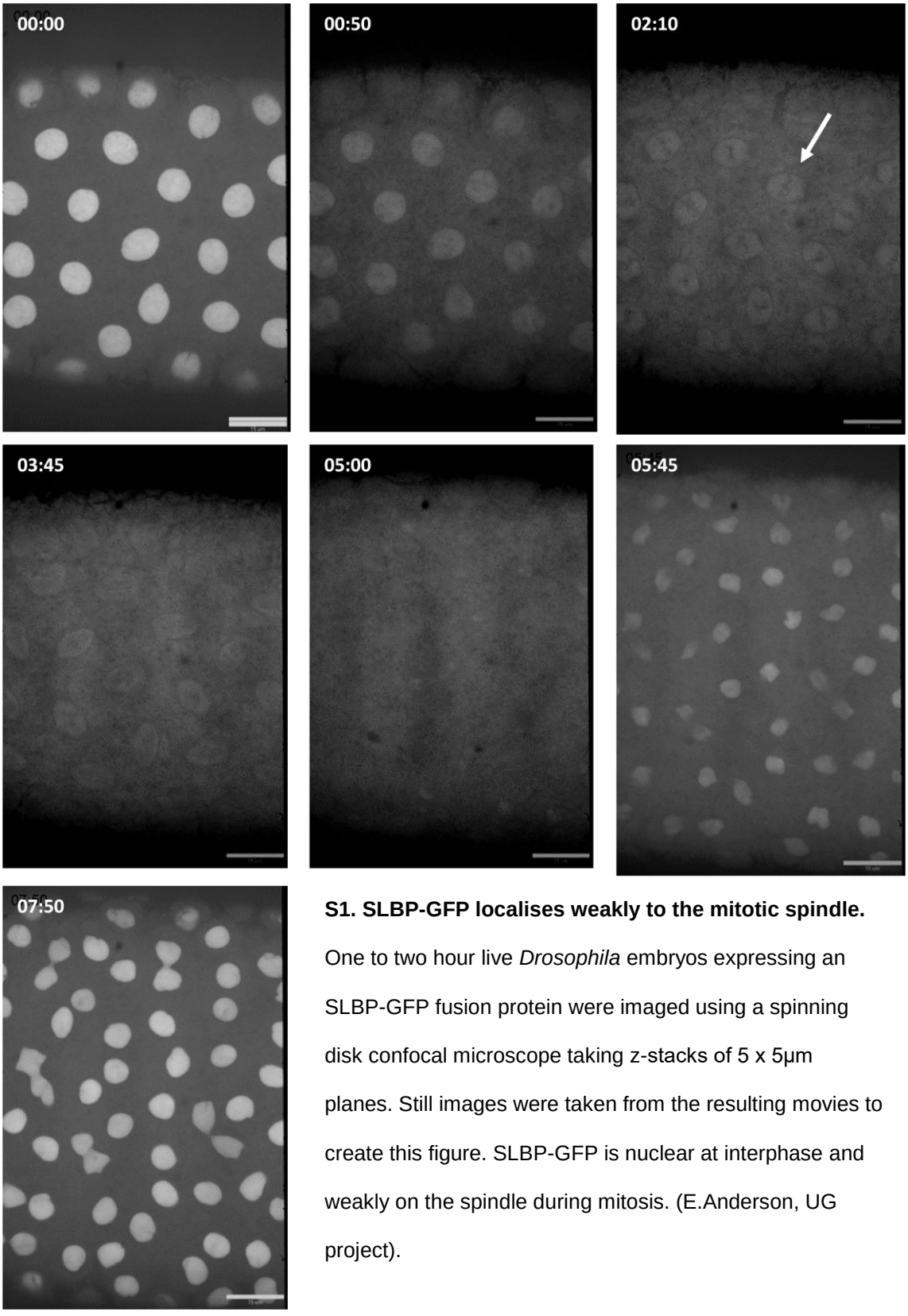
4.6. Final remarks

In summary, this project has provided evidence for the existence of previously uncharacterised mitotic functions of *Drosophila* stem loop-binding protein, SLBP. I have shown that, although SLBP does not biochemically behave as a mitotic MAP, the phenotype of embryos in which SLBP has been reduced via RNAi appears to be DNA damage response-like, without consistently occurring in the presence of DNA damage. This contradicts previous research that has attributed mitotic defects in slbp mutant/RNAi lines to the result of DNA damage caused by insufficient histone protein levels. I have also identified a group of proteins that SLBP interacts with, independently of their mRNAs; and in the absence of SLBP, mis-localisation of, at least, centrosomal interactors was observed. The interactors identified could also cause a similar phenotype to what we have observed, if their function is compromised, providing a compelling hypothesis as to how the DNA damage-like phenotype is achieved in the absence of DNA damage. It is also possible that SLBP could interact with further, unidentified proteins, which may contribute to the observed phenotype, as I may not have managed to pull down all interactors in the IP and mass spectrometry analysis. In addition, I have suggested a hypothesis for how SLBP could act on the variety of proteins described, such that in its absence we

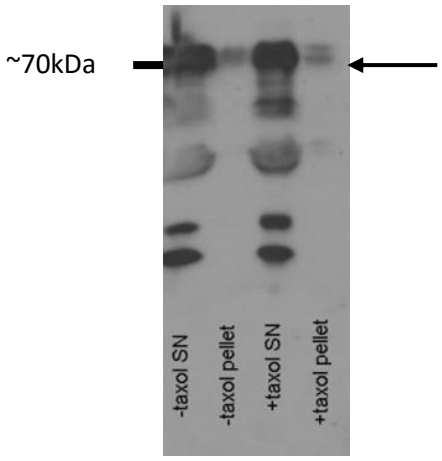
observe the mitotic defects characterised here. Although I have undertaken a preliminary investigation to prove our buffer hypothesis, results are so far inconclusive. Therefore, further study, as outlined above, is needed to develop our idea.

This work has important clinical applications for the human disease Wolf-Hirschhorn Syndrome (WHS). WHS is cause by a genetic deletion in the short arm of chromosome 4, which can encompass the Slbp gene, resulting in reduced SLBP levels in patients (Kerzendorfer *et. al.,* 2012). Therefore, understanding how this protein functions in cells is vital to investigate options for disease management.

5. Supplementary Materials



S1. SLBP-GFP localises weakly to the mitotic spindle. One to two hour live *Drosophila* embryos expressing an SLBP-GFP fusion protein were imaged using a spinning disk confocal microscope taking z-stacks of 5 x 5µm planes. Still images were taken from the resulting movies to create this figure. SLBP-GFP is nuclear at interphase and weakly on the spindle during mitosis. (E.Anderson, UG project).



S2. Biochemical analysis could not determine if SLBP-GFP associates with microtubules. Western blot of a MT co-sedimentation assay performed on 0-3h embryos expressing an SLBP-GFP fusion protein, probed for GFP. The major band representing SLBP-GFP, at ~70kDa, shows a large volume of the protein did not pellet with microtubules in the presence of taxol, as it remained in the supernatant (SN). However, a band can also be seen in the +taxol pellet (P) which would suggest some of the protein did associate. Therefore these results were inconclusive.

Fly food recipe

For 11L:

11kg Yeast

400g Glucose

400g Molasses

500g Flour

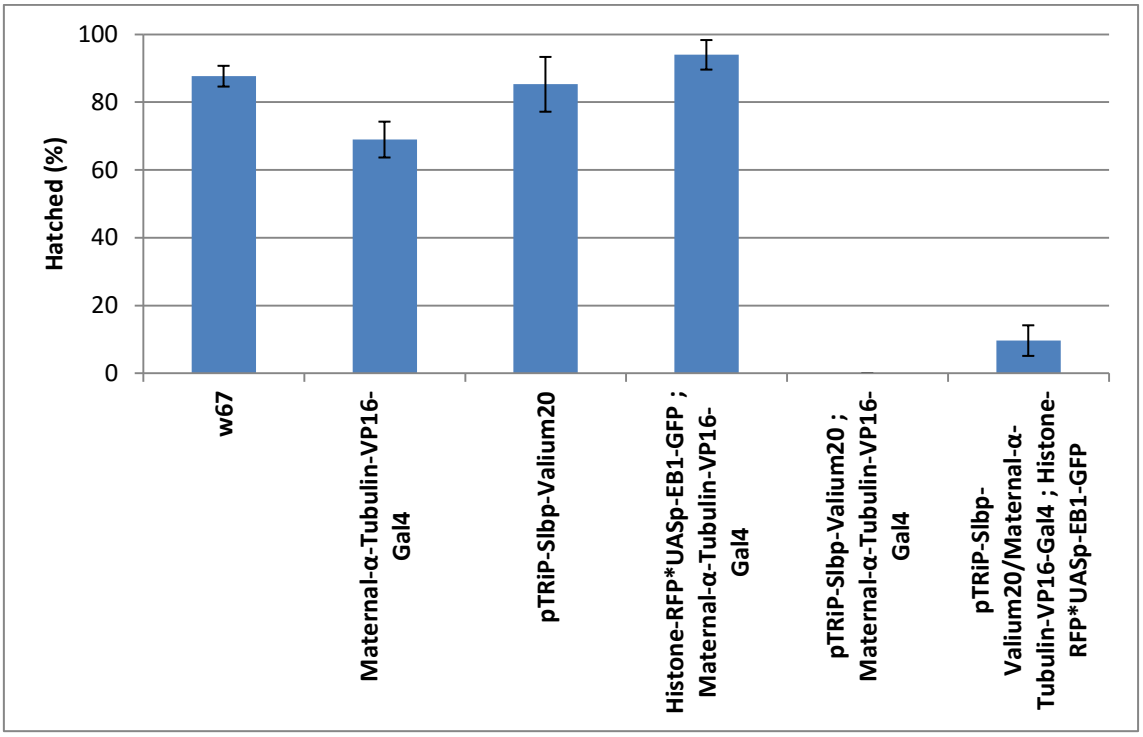
110g Agar

60ml Propionic Acid

220ml 10% Nipagin

1) Add yeast, glucose and molasses to 7L hot water and boil for 15min while mixing
2) Add flour and agar to 4L hot water, dissolve, and add to the main mix and boil for a further 15min while mixing
3) Turn heat off and cool to 60°C while constantly mixing
4) Add propionic acid and nipagin
5) Pour into bottles and vials
6) Leave to set and then plug the tops with bungs when cold

S3. Fly food recipe



S4. Hatch rate analysis. One hundred embryos, from apple juice agar plates which flies had layed on overnight, were placed on a fresh apple juice agar plate and incubated at 25°C for 48h. The number of embyros which had hatched into larvae were recorded after this time. The experiment was repeated in triplicate for each line.

S5. Full movie of live slbp-RNAi embryos co-expressing Histone-RFP and EB1-GFP transgenes, obtained on a spinning disk confocal microscope. Attached.

S6. Full movie of live control embryos co-expressing Histone-RFP and EB1-GFP transgenes, obtained on a spinning disk confocal microscope. Attached.

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